









STUDIES  
FROM  
THE ROCKEFELLER INSTITUTE  
FOR MEDICAL RESEARCH

REPRINTS  
VOLUME XVIII



NEW YORK  
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

1914

25015

*Results of the investigations conducted at The Rockefeller Institute, or elsewhere under its grants, are first reported in a variety of journals and publications. The reports are then assembled in the volumes designated Studies from The Rockefeller Institute for Medical Research, of which this is number XVIII. The Studies appear serially but at irregular intervals. The text of the original publications is in all respects followed in the Studies. The name, date, number, and pages of the journal in which each article originally appeared are printed above the title. To insure uniformity and simplicity of reference, plates and illustrations repeat the numbers used in the place of first publication.*

COPYRIGHT, 1913, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE NEW ERA PRINTING COMPANY  
41 NORTH QUEEN STREET  
LANCASTER, PA.

# CONTENTS.

## PATHOLOGY AND BACTERIOLOGY.

	PAGE.
FLEXNER, SIMON. Some Problems in Infection and Its Control	I
ROUS, PEYTON, MURPHY, JAMES B., and TYTLER, W. H. A Filterable Agent the Cause of a Second Chicken Tumor, an Osteochondrosarcoma .....	30
NOVY, F. G., PERKINS, W. A., and CHAMBERS, R. Immunization by Means of Cultures of <i>Trypanosoma lewisi</i> .....	35
MORSE, MARY ELIZABETH. The Application of the Complement-Fixation Reaction to the Diphtheria Group of Organisms .....	51
PEARCE, RICHARD M., AUSTIN, J. H., and MUSSER, JOHN H., JR. The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. III. The Changes in the Blood Following Splenectomy and Their Relation to the Production of Hemolytic Jaundice .....	59
KARSNER, HOWARD T., and PEARCE, RICHARD M. The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. IV. A Study, by the Methods of Immunology, of the Increased Resistance of the Red Blood Corpuscles after Splenectomy .....	70
PEARCE, RICHARD M., and AUSTIN, J. H. The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. V. Changes in the Endothelial Cells of the Lymph Nodes and Liver in Splenectomized Animals Receiving Hemolytic Serum .....	81
FOLIN, OTTO, KARSNER, HOWARD T., and DENIS, W. Nitrogen Retention in the Blood in Experimental Acute Nephritis of the Cat .....	90
HOWARD, C. W., and CLARK, PAUL F. Experiments on Insect Transmission of the Virus of Poliomyelitis .....	98

	PAGE.
MANWARING, WILFRED H. The Effects of Subdural Injections of Leucocytes on the Development and Course of Experimental Tuberculous Meningitis. Second Paper ..	108
NOGUCHI, HIDEYO. Cultivation of <i>Treponema calligyrum</i> (New Species) from Condylomata of Man .....	121
FLEXNER, SIMON, CLARK, PAUL F., and FRASER, FRANCIS R. Epidemic Poliomyelitis. Fourteenth Note: Passive Human Carriage of the Virus of Poliomyelitis .....	131
FLEXNER, SIMON, and NOGUCHI, HIDEYO. Experiments on the Cultivation of the Virus of Poliomyelitis. Fifteenth Note .....	136
ROUS, PEYTON, and MURPHY, JAMES B. Variations in a Chicken Sarcoma Caused by a Filterable Agent .....	138
NOGUCHI, HIDEYO, and MOORE, J. W. A Demonstration of <i>Treponema pallidum</i> in the Brain in Cases of General Paralysis .....	151
BUTTERFIELD, E. E. The Reaction between Oxygen and Hemoglobin .....	158
LAWSON, MARY ROWLEY. The Extracellular Relation of the Malarial Parasite to the Red Corpuscle, and Its Method of Securing Attachment to the External Surface of the Red Corpuscle .....	165
BRONFENBRENNER, J., and NOGUCHI, H. On the Resistance of Various Spirochætes in Cultures to the Action of Chemical and Physical Agents .....	185
MANWARING, WILFRED H. The Relation of the Leucocytic Bacteriolysin to Body Fluids .....	192
TYTLER, W. H. A Transplantable New Growth of the Fowl, Producing Cartilage and Bone .....	207
MURPHY, JAMES B. Transplantability of Tissues to the Embryo of Foreign Species. Its Bearing on Questions of Tissue Specificity and Tumor Immunity .....	223
ROUS, PEYTON. False Transitions between Normal and Cancerous Epithelium .....	235
NOGUCHI, H. Studien über den Nachweis der <i>Spirochæta pallida</i> im Zentralnervensystem bei der progressiven Paralyse und bei Tabes dorsalis .....	239

	PAGE.
FLEXNER, SIMON. The Results of the Serum Treatment in Thirteen Hundred Cases of Epidemic Meningitis .....	248
FLEXNER, SIMON, and CLARK, PAUL F. Paralysis in a Dog Simulating Poliomyelitis .....	272
BUTTERFIELD, E. E., and PEABODY, F. W. The Action of Pneumococcus on Blood .....	277
BUTTERFIELD, E. E. The Color Index and Color of the Red Blood Corpuscles .....	283

#### PHYSIOLOGY AND PHARMACOLOGY.

WOLLSTEIN, MARTHA, and MELTZER, S. J. Pneumonic Le- sions Made by Intrabronchial Insufflation of Non-Virulent Pneumococci .....	288
AUER, JOHN, and MELTZER, S. J. Der afferente Splanchnikus als Depressor .....	297
WOLLSTEIN, MARTHA, and MELTZER, S. J. The Reaction of the Lungs to the Intrabronchial Insufflation of Killed Vir- ulent Pneumococci and of Plain Sterile Bouillon .....	301
MELTZER, S. J. Simple Devices for Effective Artificial Respi- ration in Emergencies .....	306

#### CHEMISTRY.

LEVENE, P. A. The Sulphatide of the Brain .....	318
LEVENE, P. A., and LA FORGE, F. B. On Nucleases. Third Paper .....	320
LEVENE, P. A., and MEYER, G. M. On the Action of Leuco- cytes on Some Hexoses and Pentoses. Third Communi- cation. Contribution to the Mechanism of Lactic Acid Formation from Carbohydrates .....	323
LEVENE, P. A., and WEST, C. J. On Cerebronic Acid. Second Paper .....	329
LEVENE, P. A. On the Components of Sphingomyelin .....	339

#### EXPERIMENTAL SURGERY.

REL, ALEXIS. Visceral Organisms .....	341
---------------------------------------	-----

	PAGE.
CARREL, ALEXIS. Artificial Activation of the Growth <i>in Vitro</i> of Connective Tissue .....	344
INGEBRIGTSEN, RAGNVALD. Studies of the Degeneration and Regeneration of Axis Cylinders <i>in Vitro</i> .....	350
EBELING, ALBERT H. The Permanent Life of Connective Tissue Outside of the Organism .....	360

## EXPERIMENTAL BIOLOGY.

LOEB, JACQUES. The Comparative Efficiency of Weak and Strong Bases in Artificial Parthenogenesis .....	373
BEUTNER, REINHARD. Die physikalische Natur bioelektrischer Potentialdifferenzen .....	386
LOEB, JACQUES. Untersuchungen über Permeabilität und antagonistische Elektrolytwirkung nach einer neuen Methode	410
LOEB, JACQUES, and WASTENEYS, HARDOLPH. Die Oxydationsvorgänge im befruchteten und unbefruchteten See-sternei .....	450
BEUTNER, REINHARD. Einige weitere Versuche betreffend osmotische und kolloidale Quellung des Muskels.....	453
LOEB, JACQUES, and BANCROFT, F. W. The Sex of a Parthenogenetic Tadpole and Frog .....	461
BEUTNER, REINHARD. New Galvanic Phenomena .....	463
BEUTNER, REINHARD. New Electric Properties of a Semipermeable Membrane of Copper Ferrocyanide .....	472
LOEB, JACQUES, and WASTENEYS, HARDOLPH. The Relative Influence of Weak and Strong Bases upon the Rate of Oxidations in the Unfertilized Egg of the Sea Urchin ..	487

## THE HOSPITAL OF THE ROCKEFELLER INSTITUTE.

COHN, ALFRED E. On the Differences in the Effects of Stimulation of the Two Vagus Nerves on Rate and Conduction of the Dog's Heart .....	494
McCrudden, F. H., and FALES, H. L. The Nature and Origin of the Nitrogenous Compounds in the Feces in Infantilism .....	520

	<b>PAGE.</b>
McCRUDDEN, F. H., and FALES, H. L. The Cause of the Excessive Calcium Excretion through the Feces in Infantilism	524
PEABODY, FRANCIS W. Studies of the Inorganic Metabolism in Pneumonia with Especial Reference to Calcium and Magnesium .....	529
SWIFT, HOMER F. Absorption of Arsenic Following Intramuscular Injections of Salvarsan and Neosalvarsan ..	541
McCRUDDEN, F. H., and FALES, H. L. Intestinal Absorption in Infantilism .....	547
McCRUDDEN, F. H., and FALES, H. L. The Cause of the Failure to Develop in Infantilism .....	550
ROBINSON, G. CANBY. The Influence of the Vagus Nerves on the Faradized Auricles in the Dog's Heart .....	554
DRAPER, GEORGE, and HANFORD, JOHN M. Experiments on the Transmission of Scarlet Fever to the Lower Monkeys	569
COHN, ALFRED E. The Post-Mortem Examination of Horses' Hearts from Cases of Auricular Fibrillation .....	579
COHN, ALFRED E. Observations on Injection Specimens of the Conduction System in Ox Hearts .....	585





## SOME PROBLEMS IN INFECTION AND ITS CONTROL.\*

By SIMON FLEXNER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.*)

I experience a high sense of honour on this occasion, with which is mingled no less trepidation in view of the master in whose memory this lectureship was founded, and the great names that in the past have been linked with the post I am to-day asked to fill.

### HUXLEY AND THE EARLY DAYS OF BACTERIOLOGY.

I must believe that Huxley would have felt a deep interest in the theme which I have chosen to discuss before you, and would have found in its intrinsic importance a compensation for any shortcoming that may appear in the presentation. For Huxley evinced a penetrating appreciation of that branch of biological science that has come to be called bacteriology, and as President of the British Association in 1870 devoted the occasion of his address to an illuminating examination of the doctrine of abiogenesis, or spontaneous generation, *versus* the doctrine of biogenesis or descent from living ancestors. This subject, long holding a merely academic interest, had become in the two decades immediately preceding the ground over which the conflict raged and out of which was to emerge the modern science of microbiology.

While Huxley clearly pointed out that Redi in the seventeenth century and Spallanzani in the eighteenth had delivered the first telling blows that later, through Pasteur, led to the overwhelming defeat of the spontaneous generationists and the establishment on an indisputable basis of the extrinsic origin of the contagious and infectious diseases, he did not fail to perceive in the discoveries just

\* The Huxley Lecture, delivered in the Charing Cross Hospital School of Medicine, London, October 31, 1912.

being made in reference to fermentation, putrefaction, and certain fungus and other diseases of insects, the herald of the new science that was to throw its protecting mantle, not about man alone, but about all the higher animals, and even about the plants, in order that the useful and indispensable should be protected from that inevitable contest in nature between higher and lower forms of life which constitutes disease and leads to premature decay and ruthless destruction.

#### OBSTACLES IN THE INVESTIGATION OF DISEASE: THE CONQUEST OF SYPHILIS.

Bacteriology has, up to now, distributed its favours unequally, but we must not be daunted by this circumstance. It has yielded in some instances knowledge of diseases of small, and withheld in others knowledge of diseases of great, importance. In respect to the common and highly contagious diseases, measles and scarlet fever, for example, progress has been slight. A ray of hope has been cast upon this quest by the announcement<sup>1</sup> that measles can be caused in the monkey by inoculation of infected blood, but this awaits certain confirmation. Similar announcements have been made recently regarding scarlet fever.<sup>2</sup> Since a flood of knowledge has always suddenly flowed from the successful transmission of an obscure disease to the lower animals these reports have been viewed with eager expectation. In the case of scarlet fever I fear the expectation is not yet to be realised. We<sup>3</sup> spent last winter in the study of this subject and failed completely to infect or produce scarlet fever in a wide variety of lower monkeys. Possibly, but not certainly, the higher anthropoid ape, which is still less removed from the human species, is subject to inoculation.<sup>4</sup>

<sup>1</sup> Anderson and Goldberger: Bulletin of the United States Public Health and Marine Hospital Service, 1911, No. 62.

<sup>2</sup> Cantacuzène: Comptes rendus de la Société de biologie, 1911, vol. lxx., p. 403. Bernhardt: Deutsche medizinische Wochenschrift, 1911, vol. xxxvii., pp. 791 and 1062; Centralblatt für Bakteriologie, Parasitenkunde, und Infektionskrankheiten, Abteilung 1, Referate, Supplement, 1911, 1, 27.

<sup>3</sup> Draper, George: Unpublished studies.

<sup>4</sup> Landsteiner, Levaditi, and Prasek: Annales de l'Institut Pasteur, 1911, vol. xxv., p. 754.

The path of success in relation to the refractory diseases is marked by heavy obstacles, but it must be travelled none the less. How often, indeed, has crowning success come to the brave, thoughtful, and adventurous when all but an expiring glimmer of hope had gone! Witness in this connexion the sudden conquest of syphilis, in which the initial victory was won when it was ascertained that anthropoid apes can be infected experimentally. There followed in rapid succession the discovery of the causative spirochæte, the Wassermann clinical test, and the fabulous drug salvarsan, the usefulness of which outruns the wide bounds of syphilis itself.

But even after such a victory the drama had not come to an end. The spirochætal cause could now be discovered regularly where it had been as constantly missed before; doubts and misbeliefs in it were quickly yielding before the rapidly accumulating evidence; but the micro-organism itself resisted all attempts at artificial cultivation. That the spirochæte is a parasite nicely adjusted to living tissues was clear from the difficulties surrounding the experimental inoculation of animals. Now this act also has been played.<sup>5</sup> The *pallida* has yielded to artificial culture by Noguchi, and the method sufficing for it has suddenly exposed the whole class of disease-producing spirochætæ and some innocent species as well to cultivation and exploitation under laboratory conditions. It is obvious that the more nicely a parasitic organism is adjusted to its host the more difficult it will be to cultivate it outside the host, and the more quickly it will lose in culture its pathogenic power. The *pallida*, which for so long resisted the efforts to transmit it artificially to animals and then to cultivate it outside *in vitro*, loses, after a few generations, as was to be expected, its disease-producing virulence, while the blood parasites of relapsing and tick fevers in man and spirillosis in fowls, which are less strictly parasitic and pass a stage of their life in biting insects, retain this power for many generations. In turn, the culture of the *pallida* has yielded luetin that by causing a local allergic or hypersensitive skin reaction has provided clinical medicine with a new means of detecting latent luetic infection.

<sup>5</sup> Noguchi, *Journal of Experimental Medicine*, 1911, vol. xiv., p. 99; 1912, vol. xv., p. 90; 1911, vol. xiv., p. 557; 1912, vol. xvi., p. 199.

## POLIOMYELITIS.

With this introduction to the more general theme of the hour, I shall invite you to follow with me somewhat minutely the biological investigation of a disease that is still claiming the absorbed attention of both physicians and people—namely, poliomyelitis, or infantile paralysis. The disease has just been making the rounds of the world, coming as a very unwelcome intruder to many different countries. Until the recent pandemic it was surrounded with mystery and fortified by superstition. It is the story of the working out of the natural history of poliomyelitis, now elucidated in many ways, that I propose to tell you. I have been led to choose this particular disease as my theme both because it has claimed much of my attention during the past several years, and because it illustrates admirably certain general truths to which I desire to call your attention.

Poliomyelitis has been endemic in Northern Europe for many years, but it is only five years since it started on that unique, and as yet unexplained, movement that has carried it around the globe. In America there is no previous history of a general prevalence or epidemic, although local outbreaks of infantile paralysis have from time to time arisen. Some significance attaches to the fact that the first two foci of the present epidemic—I say present, because since 1907 the disease has prevailed severely each summer and autumn at some places in the United States and Canada—arose in the Atlantic coast cities and in the State of Minnesota in the Middle West. The former receive the mass of emigrant population from Europe, and the latter, secondarily, the large contingent of Scandinavian emigrants. The imposition of the infection upon America can thus be accounted for; but no explanation is afforded of the many years of immunity while Scandinavians were constantly arriving, and for the penetration of the disease to other European countries and to far-distant parts of the world. However, within the pandemical period the disease has taken on new activity in Norway and Sweden, and as recently as 1911 the latter country has suffered a severe visitation.

EXPERIMENTAL INVESTIGATION OF THE DISEASE: ROUTES OF  
INFECTION.

On clinical grounds Scandinavian observers<sup>6</sup> had recognised the essentially infectious nature of poliomyelitis and had followed the evolution of the outbreaks and traced the connexion between many of the cases. They became the defenders of the notion of human carriage, and by establishing certain unusual clinical forms of the disease—such as the meningeal and abortive—placed this idea on firm ground. The notion was further extended to include healthy carriers of the infection who act as intermediaries between the actively ill and the new victims of infection. These views have all alike been treated with more or less scepticism by the medical profession; in how far they have come to be supported by later acquisitions of knowledge will appear.

Apart, then, from these deductions, disputed and disputable, because not supported by certain tests, five years ago the mystery of the disease was wholly unfathomed. The outlook was suddenly brightened when Landsteiner and Popper in 1909<sup>7</sup> announced the successful transmission of poliomyelitis to monkeys, but the high hopes raised were as quickly damped by the failure to propagate the experimental disease beyond the first generation. This obstacle was immediately removed when intracerebral was substituted for intraperitoneal inoculation, as was done by Lewis and myself,<sup>8</sup> and by Landsteiner and Levaditi.<sup>9</sup> By this means the disease could be, and has been, transmitted through an indefinite number of monkeys. The inoculating matter is, first, the sterile spinal human case, and afterwards, the spinal cord of *para*

The choice of the intracerebral route as superic



\* Wickman: Beiträge zur Kenntnis der Heine-Medinschen

1907.

<sup>7</sup> Landsteiner and Popper: Zeitschrift für Immunitätsforschung, Originale, 1909, vol. ii., p. 377.

<sup>8</sup> Flexner and Lewis: Journal of the American Medical Association, 1909, vol. liii., p. 1639; Journal of Experimental Medicine, 1910, vol. xii., p. 227. Flexner: Journal of the American Medical Association, 1910, vol. lv., p. 1105. Flexner and Clark: Ibid., 1911, vol. lvii., p. 1685.

<sup>9</sup> Landsteiner and Levaditi: Comptes rendus de la Société de biologie, 1909, vol. lxxvii., p. 592. For general bibliography consult Römer, Die epidemische Kinderlähmung, Berlin, 1911.

peritoneal was not haphazard. All the severe effects of poliomyelitis are inflicted on the nervous system, and upon reflection this fact at once suggested that the parasitic cause of the disease must find favourable conditions for multiplication within the nervous tissues. When the material carrying the germ is put first into the peritoneal cavity it must traverse the blood before it can reach the nervous system, and the blood, as we know, has the power to destroy many forms of germ life. It could, of course, also be reasoned that the specific parasite, in nature, cannot enter the nervous tissues directly, but must use some external route to reach them, and it must, therefore, be capable of surviving outside the brain and spinal cord; and it could be further reasoned that an inoculation into a more accessible part of the body than the brain and spinal cord should be effective, and if effective would bring stronger proof of the actual existence of a parasite in the inoculated matter. This reasoning is unconvincing for two causes: first, the monkey is not naturally subject to poliomyelitis, and is, therefore, presumably more difficult to infect at all than is man, so that what may suffice to cause infection in man may fail in the monkey; and secondly, it might be possible for pathogenic microbes to reach the central nervous system even in man without entering the blood at all, so that in nature the infectious cause of poliomyelitis might avoid the blood altogether. That this possibility really exists has been proved by experiment as we shall see. Doubtless the first material inoculated into the abdominal cavity carried besides the living parasites toxic or other injurious substances that promoted infection in the monkey; but when the nervous tissues of the monkey were similarly injected, being less harmful, the inoculation failed. Bacteriology contains many instances of similar, and apparently of paradoxical, nature.

The discrepancy has been further elucidated as will soon appear, but in the meantime it is desirable to inquire whether still other routes of infection do exist for the monkey. Since nervous tissue is favourable to the parasite it was injected into large nerves—such as the sciatic—in order to ascertain whether these furnished a suitable medium of propagation. The parasite grows along the nerve until the spinal cord is reached and produces injury of the cord first at the point of entrance before it extends to and attacks other parts.

The injection into the nerve causes no symptoms, but paralysis of the innervated muscles appears after the lapse of a time sufficient for the necessary multiplication of the parasite and its passage into the spinal cord.

Meanwhile the inoculated monkey shows no other signs of illness, and no other organ is severely affected; the injury is centred upon the nervous tissues. And not only does the parasite grow or flow along the nerve, but it ascends along the spinal cord from lower to higher levels and eventually reaches the medulla and brain. At last the centres governing respiration are involved and death by paralysis ensues.

#### PATHIOLOGICAL EFFECTS ON SPINAL CORD AND BRAIN.

We have now been able to arrive at several important conclusions. The monkey can be made regularly to develop an experimental disease agreeing in all essential respects with poliomyelitis in man. Inoculation is necessary, since keeping healthy and paralysed monkeys together does not lead to infection. The parasitic cause of the disease can traverse the blood, in the monkey, to reach the central nervous organs, but with difficulty, while it easily traverses the peripheral nerves. That the natural, spontaneous disease, so called, in man and the induced disease in monkeys are very alike is further shown by microscopic study of the spinal cord and brain, which exhibit changes that are identical.

The pathological effects are of two kinds: injury to nerve cells, not in the anterior grey matter alone, but in the posterior grey matter of the spinal cord and in the intervertebral ganglia, medulla, and brain; and cellular invasion of the pia-arachnoidal membrane of the spinal cord and medulla that follow the blood-vessels into these parts and pass into the adjacent grey and white matter. The altered vessels permit an escape of albuminous fluid and blood cells into the meshes of the membrane, where they mingle with the cerebro-spinal liquid, and into the spaces in the tissue composing the solid white and grey matter. Sometimes the nerve cells, sometimes the meninges, vessels and supporting tissues suffer most. When the nerve cells are extensively injured the paralysis is marked; when the meninges are much affected the symptoms are like those of



meningitis. The virus of poliomyelitis displays a high affinity for nervous tissues, but it is the wide involvement of the nutritive vascular system in the pathological process that subjects the sensitive nerve cells to so high a degree of injury and destruction.

#### THE INFECTIOUS AGENT OF POLIOMYELITIS.

The microscopical conditions we observed in the course of our experiments were suggestive of two things: first, the nature of the parasite itself; and secondly, the process of generation of the effects or lesions themselves. Up to this time no definite parasite could be detected in the nervous tissues either in human beings or monkeys, nor was anything of the kind found in the blood or other organs. The scarcity of polynuclear leucocytes in the altered cerebro-spinal liquid and spinal cord itself spoke against a simple bacterial parasite. The large number of mononuclear cells spoke rather for a protozoal parasite. Neither could be found, although the most varied methods of staining and cultivation were employed. There remained the possibility of the parasite being invisible or ultra-microscopic and filterable. This it proved to be, for when a portion of the spinal cord of a recently paralysed monkey was made into an emulsion with sterile distilled water or simple saline solution and then centrifuged to remove the coarse suspended matter and afterwards pressed through a Berkefeld earthenware filter—which excludes ordinary cells, bacteria, and protozoa—the clear liquid resulting was still capable of transmitting the disease. The activity of the filtrate is very great, since a fraction of a cubic centimetre still suffices to cause paralysis and death. The only distinction to be noted between the action of corresponding amounts of the emulsion and filtered fluid is that the former acts more quickly, as would be expected from the fact that it contains a greater number of the invisible organisms. This difference is soon compensated by the multiplication of those in the filtrate, so that the end result is the same. By employing somewhat greater quantities of the filtrate for inoculation the incubation period of the disease can be made the same as that following the use of the emulsion. The disparity is strictly a quantitative one, since the filters retain a part of the minute organisms in their pores and thus reduce the number escaping with the

filtrate. The greater the quantity of protein matter present in the fluid the fewer the parasites that pass the filter, and merely because the large protein molecules themselves tend to be held in the pores and thus render them impervious for the minute organisms. For this reason, also, fluids containing small numbers of the filterable parasites, but still sufficient to cause infection in the crude state, may fail, when filtered, to produce disease merely because those retained by the filter so far reduce the numbers as to bring them below the surely infecting dose. This reduction sometimes leads to another effect—namely, the slight degree of infection that forms the starting point of active immunisation. By building upon such a beginning a high and enduring state of immunity has been achieved.

#### FILTERABLE VIRUSES.

The first filterable parasite was discovered by Loeffler 14 years ago, in the fluid lymph obtained from the vesicles of cattle suffering from foot and mouth disease. At the present time 18 diseases are known that are believed on good ground to be caused by this class of minute living organisms. One alone among them is on the verge of visibility—the parasite causing pleuro-pneumonia of cattle. It alone has certainly been obtained in artificial culture. The methods of artificial cultivation need still to be worked out; and once they are discovered it is a safe prediction that control over the diseases produced by ultra-microscopic parasites will be quickly increased. The degree of infectivity of certain of the parasites—or viruses as they are also called—is almost fabulous. One-thousandth of a cubic centimetre of a filtered 2.5 per cent. suspension of a spinal cord of a paralysed monkey suffices to cause infection and paralysis in another monkey,  $1/20,000$ th of a cubic centimetre of infected lymph suffices to produce foot and mouth disease in a healthy calf, and the blood of fowl suffering from chicken plague is still active after being diluted with water 1000 million times.

Three affections of human beings are contained among the 18 diseases caused by filterable viruses: they are yellow fever, dengue, and poliomyelitis. With one exception—mosaic disease of tobacco—the remaining 14 are maladies of domestic animals, and include among them foot and mouth disease, horse sickness, cattle plague,

sheep pox, rabies, vaccinia, hog cholera, and chicken plague. We can at present form no reliable conception of the biology of this class of parasite, although the virus of pleuro-pneumonia shows affinities with the bacteria, while that of yellow fever, that passes a stage of its existence in mosquitoes, probably belongs to the protozoa. It should be remembered that we possess no criterion of their presence other than the power to produce infection. Probably the list of these pathogenic parasites would be increased if methods were known for testing their symbiotic relations or coöperative effects with the usual bacteria and protozoa. Rous's<sup>10</sup> discovery of a filterable agent that causes sarcomatous tumours in the fowl has opened up new fields to exploration. We can make a rough guess as to their sizes since some pass through thick filters the pores of which are smallest, while others pass the more porous filters with larger interstices only. Were the viruses as large as one-fifth the size of the influenza bacillus they would be beyond visibility with the most powerful optical system of the modern microscope. The dark-field microscope and the instrument devised for employing, for photographic purposes, the ultra-violet rays of the spectrum, that has doubled the potential power of the microscope, have failed to bring them into view. On the whole they resist drying well and show considerable resistance to disinfecting agents.

The ultra-microscopic viruses employ no single means of effecting entrance into the body. Some utilise insects to inject them into the blood. Mosquitoes inoculate the parasites of yellow fever and of dengue in man, and among animals the virus of horse sickness; while flies inject the virus of pappataci fever, and worms and other insects, through mere contact with infected and then with uninfected tobacco plants, disseminate the parasite of mosaic disease. The viruses of rabies, vaccinia, and fowl pox gain entrance through skin wounds, those of hog cholera, foot and mouth disease, and chicken plague by swallowing, while the parasites of variola and of pleuro-pneumonia are inhaled with air. These are the main avenues, but not the sole routes of infection, since viruses that ordinarily enter the body by the respiratory mucous membrane may occasionally enter through a skin abrasion, &c.

<sup>10</sup> Rous: *Journal of the American Medical Assn.*, 1911, vol. lvi., p. 198.

It is significant that upon recovery from this class of infections a high and enduring degree of immunity is left behind. We have no knowledge of toxic substances, in the common sense, being produced by the filterable viruses, and therefore know nothing of the formation of antitoxins or bodies that neutralise poisons. The principles upon which the immunity depends appear to be chiefly microbicidal or substances that act directly upon the living parasites and destroy them. In some instances it has been possible to produce an actively immune state without at the same time causing severe disease, by employing for inoculation modified and weakened viruses, and viruses combined with immune sera carrying the corresponding microbicidal substances. Once a certain active immunity is obtained it can be heightened by repeated injections of more active materials until a high degree is achieved. In the same manner immune animals that have recovered from disease are capable of having this immunity reinforced by subsequent injections of the active virus. Blood taken from the immune animals has been employed in practice in two ways: to protect for a brief period exposed animals from acquiring infection, and to bring about an actively immune state through inoculation with adjusted mixtures of virus and corresponding immune serum. The injection of viruses into animals not themselves subject to infection has, in a few instances, yielded immune sera. In this way a serum for foot and mouth disease has been prepared in the horse. Speaking generally, homologous sera are more active than heterologous, or, in other words, an immune cattle serum will act better in cattle than will immune horse serum; but curative sera in a real sense have not yet been produced for this class of diseases.

#### PROPERTIES OF VIRUS OF POLIOMYELITIS.

It is of great interest to determine the correspondence between the general data I have just reviewed and the special facts of poliomyelitis which have been shown to arise in consequence of an invasion of the nervous tissue by an ultra-microscopic or filterable virus.

We may proceed to check off rapidly the main facts. The virus stands midway in point of size between the finest and coarsest examples. It passes readily through the more coarse and slightly

through the finest filters. It is highly resistant to drying, light, and chemical action. In dust, especially within protein matter, it survives weeks and months; in diffuse daylight indefinitely, and resists the action of pure glycerine and carbolic acid in 0.5 per cent. solution for many months. When animal tissues containing the virus suffer softening and disintegration or disorganisation by mould the virus survives.

Recovery from poliomyelitis in man and monkey is attended and produced by an immunisation of the body. During this process microbicidal substances appear in the blood that are capable of neutralising the active virus. This acquired immunity has, in the monkey, been reinforced by subsequent injection of large quantities of the living virus. Active immunity can be achieved by first injecting minute and later large amounts of the virus; and an adjusted mixture of immune serum and active virus will confer a beginning low active immunity capable of being heightened.

Certain alien large animals, among which the horse and sheep are especially worth mentioning, are subject to immunisation through injections of emulsions of the spinal cord and brain of paralysed monkeys, and can thus be made to yield sera possessing microbicidal power and capable of conferring, as do human and monkey immune sera, a degree of passive immunity. Thus far no immunising effect has been accomplished with the dead virus. Unless some growth and multiplication take place no immunity arises.

#### MODE OF INFECTION.

These facts show a close correspondence between the properties of the virus of poliomyelitis and those of the ultra-microscopic organisms in general. There remain to be considered the data bearing upon the manner of entrance of the poliomyelitic virus into the body or, in other words, upon the mode of infection.

Analogy with other diseases produced by filterable viruses excludes no one of the possible modes, since their manner of entrance is widely varied, as we have seen. This question is of the utmost importance, since with all diseases prevention is far better than the most perfect cure, and for poliomyelitis there exists at present no specific or true curative treatment. Moreover, for the most part

when the disease is first recognised it has already caused irreparable damage, and though the more general examination of the spinal fluid obtained by means of lumbar puncture for purposes of diagnosis may possibly lead to a much earlier recognition of the disease, yet its prevention will always remain the result to be aimed at. It is quite certain that an understanding of the mode of infection would lead inevitably to the framing of measures of prevention that with reasonable certainty could be expected to exercise control over the epidemic spread.

Two answers may be returned to the question: one based upon observation of human cases of poliomyelitis, and the other based upon experimental tests arranged to elicit specific replies. The first answer cannot achieve anything higher than strong probability; the second, to be valid, must explain the phenomena attending the human infection as well as those of the experimental disease. We are asked to account for certain data, of which the following is a brief statement.

Epidemic poliomyelitis is pre-eminently a disease of early childhood and finds the highest percentage of its victims in the first five years of life, but does not wholly spare older children or even adults. It is admittedly infectious, and while it is true that many more instances of single than of multiple cases occur, yet multiple ones are not by any means rare. The prevailing views on this topic are being modified rapidly by the recognition of the abortive and ambulant examples of the disease. The period of greatest prevalence is during the months of August, September, and October in the northern hemisphere and the corresponding months in the southern hemisphere, but the epidemic begins in the early spring and summer months, and the disease does not wholly disappear during the winter months. It does not, therefore, necessarily die out at any period of the year. In endeavouring to trace the channel of entrance of the virus into the body certain facts regarding its distribution in the body should be recapitulated and considered.

The infectious agent of poliomyelitis attacks chiefly the central nervous system. Indeed, it has been detected regularly in the spinal cord and brain, and in the mesenteric lymph nodes among all the internal organs. It has also been detected in the mucous membrane

of the nose and throat, and in the mucous secretions of this membrane, and in the mucous secretions of the stomach, and the small and large intestine. The virus has not been detected in such important organs as the spleen, kidneys, liver, or bone marrow. The fact is significant, but in attempting to interpret it account should be taken of the circumstance that at present we possess one means only of detecting the virus, and that is its transmission to monkeys, in which it produces characteristic paralyses and anatomical changes. On this account small quantities of the virus may conceivably escape discovery. However, the conclusion is none the less inevitable that detectable amounts of the poliomyelitic virus exist only in the few situations and organs mentioned. The distribution of the virus is identical in human beings, the subjects of the so-called spontaneous poliomyelitis, and in monkeys, in which the experimental affection is produced. Nor does it matter how the experimental inoculation is accomplished, and whether the virus is introduced by injection into the brain or large nerves or subcutaneous tissue or peritoneum, or whether it is merely applied to the nasal mucous membrane, which, it should be emphasized, next to direct intracerebral injection, affords the surest means of causing the experimental disease. In whatever way the infection is produced purposely, the distribution of the virus in infected monkeys is the same as in infected human beings.

The virus is one that is not known to increase aside from the infected body, and hence in order that it shall be capable of propagating poliomyelitis it must secure a means of escape from the infected animal. The escape is now known to occur along with the secretions of the nose and throat and the discharges from the intestine. We are obliged, therefore, to ask ourselves what the means are by which the virus confined within the interior reaches these external surfaces of the body.

#### PASSAGE (IN INFECTED ANIMALS) OF VIRUS INTO NOSE, THROAT, AND INTESTINES.

Let us begin by disregarding for the moment the essential point of the way in which the virus probably enters the body in infected human beings, and give our attention to the way in which it escapes in the infected monkey into the nose, throat, and intestines.

We may first consider the instance in which the virus is deposited in the brain, in which it becomes sealed, as it were, and cut off apparently from the exterior of the body. Having been injected into the brain, the infectious micro-organism constituting the virus multiplies both within and about the brain tissue at the site of inoculation. As multiplication progresses, the virus leaves the original site of injection and wanders through adjacent and distant parts of the central nervous tissues, becoming implanted in the medulla, the spinal cord, and the intervertebral ganglia, as well as reaching the pia-arachnoidal membranes, or meninges, in which it also spreads. Ultimately, when the virus becomes sufficient in amount, it brings about anatomical changes in the nervous system, one of the results of which is paralysis. The period intervening between the inoculation and the appearance of paralytic symptoms may be as brief as two or three days, or as long as three, four, or five weeks. The great disparity in this period depends upon the amount and quality of the virus, as well as the degree of resistance of the inoculated monkey.

The virus which has found its way to the meninges does not long remain in the cerebro-spinal fluid, with which it escapes in part into the blood, where it does not appear to undergo any further increase in amount, and indeed seems even incapable of surviving for long. A part also of the virus contained within the cerebral fluid escapes regularly by way of the lymphatic channels surrounding the short nerves of smell that pass from the olfactory lobes of the brain to the mucous membrane of the nose. It has long been known that there is an intimate connexion between the lymphatic vessels of the nasal mucous membrane and the lymphatic spaces of the pia-arachnoidal membrane.

The virus once having gained the mucous membrane of the nose may even escape into the mucus secretion, with which it is carried into the mouth, and in part swallowed, or it may become established in the substance of the nasal membrane, where it undergoes subsequent multiplication and increase. As a matter of fact both these things occur. The virus escapes with the secretions partly externally to the infected body, and a part of it is swallowed with the secretions themselves, while a persistent infection of the secretions



is maintained by means of the increase that takes place in the membrane itself. In this way is assured the escape of the virus directly into external nature, as well as the contamination of the gastrointestinal cavity, with the discharges of which it becomes commingled.

Once implanted upon the intestine multiplication not improbably continues for a time, and another source of invasion of the body is thus afforded the parasite. From the intestine it reaches in some amount the mesenteric lymph nodes, and thus enables us to account for the occurrence of the virus in those lymphatic nodes which thus form a notable exception to the general internal organs of the body.

#### SITE OF ENTRY AND EXIT OF VIRUS IN MAN.

We have now followed the route by which the poliomyelitic virus implanted within the apparently closed cavity of the skull reaches the exterior of the body. It is obvious that in the spontaneous form of the infection in man no such mode of introduction of the virus can occur. The virus must indeed enter the human body by some external channel, after which it seeks and becomes implanted upon the central nervous system. It is known that in monkeys the virus is incapable of passing the barrier of the unbroken or slightly abraded skin, of being taken up from the stomach or intestine unless the functions of these organs are previously disturbed and arrested by opium, and it is further known that it traverses with difficulty, or even not at all, the substance of the lungs. On the other hand, it is established that the virus passes with readiness and constancy from the intact, or practically intact, mucous membrane of the nose to the central nervous system.

To illustrate this point I wish to describe briefly an experiment. The spinal cord of a paralysed monkey always contains the virus we are considering. If a camel's hair pencil or pledget of cotton is covered with some of the broken-up tissue of such a cord and painted upon the mucous membrane of rhesus monkeys these animals will develop in due time the paralysis and other symptoms of poliomyelitis. Hence the virus enters the body from this surface, even though no gross injury has been inflicted upon the membrane.

We should now ask ourselves if the virus actually ascends to the brain by the direct path of the olfactory nerves or indirectly after first entering the blood. This is the same question that has been buffeted about in regard to epidemic meningitis. The meningococcus is found in the nasal mucus of persons in contact with cases of meningitis and in the sick themselves. It is not disputed that the meningococcus settles on this membrane, but opinion is divided as to whether it goes at once to the membranes of the brain or first penetrates into the blood. To produce meningitis in monkeys it does not suffice to inoculate the nasal membrane; the meningococci must be injected into the meninges themselves. But so inoculated they escape in part along the nerves of smell into the nose. The virus of poliomyelitis is so active that implantation in the nose does suffice to cause infection. If a monkey is sacrificed about 48 hours after an intranasal inoculation and the brain and spinal cord are removed and then the olfactory lobes, portions of the medulla and spinal cord are separately inoculated into other monkeys, infection is produced by the olfactory lobes alone, since in this brief period the virus has not yet reached other and more distant parts of the nervous organs.

Were the virus distributed by the blood the medulla and spinal cord would have become infective rather than the olfactory lobes, since they exhibit a greater selective affinity for the parasite. The conclusion is unavoidable that the virus ascends by the nerves of smell to the brain, multiplies first in and about the olfactory lobes, and in time passes, as I believe, into the cerebro-spinal liquid which carries it to all parts of the nervous organs. We have already learned that the virus can pass along a large nerve, such as the sciatic, which carries it first to the lumbar cord, whence it ascends to higher levels; we need not, therefore, be astonished to find that it can wander along the olfactory nerves and then descend to lower levels. The large peripheral nerves are prevented anatomically from becoming infected in nature, while the small olfactory filaments are advantageously placed to act as the means of transportation.

Hence the view I desire to place before you: that the nasal mucous membrane is the site both of ingress and egress of the virus of

poliomyelitis in man. Support for this view is found also in the study of the microscopic changes in the meninges and the central nervous tissues. Since the virus survives in the dried state it may be carried in dust; and in one instance it has been detected in sweepings from the room occupied by a person ill with poliomyelitis.<sup>11</sup> Its distribution as spray in coughing and speaking is readily accomplished, and by this means both active cases and passive carriers may conceivably be produced.

Still one link in the chain of causation of poliomyelitis as here outlined remained to be forged. The clinical evidence is strong in the suggestion that human carriers of the poliomyelitic virus exist. The virus has now been detected in the secretions of the nose, throat, and intestine of persons suffering from abortive or ambulant attacks of poliomyelitis.<sup>12</sup> The unrecognized examples of the abortive disease play a highly important part in the dissemination of the virus, through which the area of infection is extended, and the number of the attacked increased.

A similar part has been accorded by clinical observation to the healthy virus carrier, and the healthy carrier is the last to be detected, and his existence confirmed experimentally. The obstacles in the way of this confirmation are considerable but not insuperable. It is to be remembered that we possess no means of discovering the virus except that of animal inoculation. Should the experimental results arising from the inoculation of the secretions of the nose and throat of such healthy carriers be confirmed the evidence for the mode of infection as here outlined would be complete. The membrane of the nose and throat is far more vulnerable in young individuals, whence arises the greater prevalence during childhood of those diseases the causes of which seek this avenue of entrance into the body. Among them are included diphtheria, measles, scarlet fever, and meningitis.

#### EXPERIMENTS AS TO INSECTS CARRYING THE INFECTION.

Would the establishment of the respiratory avenue of entrance of the virus exclude all other modes of possible infection? By no

<sup>11</sup> Neustaedter and Thro: *New York Medical Journal*, 1911.

<sup>12</sup> Kling, Wernstedt, and Pettersson: *Zeitschrift für Immunitätsforschung und experimentelle Therapie*, Orig., 1912, Band xii., p. 316.

means. Plague bacilli are known to be inoculated into man by rat fleas; but the pneumonic form of the plague is admittedly caused by respiratory inoculation. Diphtheria arises upon the mucous membrane of the throat, but can develop in a wound of the skin; the virus of small-pox enters by way of the throat and nose, but can enter by a skin abrasion; the virus of foot and mouth disease is taken in with food, but produces infection when injected into the skin. Hence at the moment, while knowledge is still recent and not yet perfect, the too absolute adherence to one point of view is to be avoided.

Indeed, the preponderance of cases in the late summer and autumn months early suggested an insect carrier of the infection. House flies can act as passive contaminators, since the virus survives upon the body and within the gullet of these insects. It has not proven possible thus far to infect the common varieties of mosquito, and the body and head louse, while success has resulted in one instance in producing infection in bed bugs which were made to feed upon the blood of inoculated monkeys. The virus remained alive within these insects for a period of many days. The inoculation of monkeys with a filtrate prepared from them gave rise to characteristic paralysis and anatomical lesions. This result is significant, since it shows that insects are capable of taking up the virus from the blood, where it exists in minimal quantities, and in harbouring it for a considerable period in an active state; but it does not show that multiplication occurs within them or that in nature they act as the agents of inoculation. A tentative announcement has been made recently by Rosenau<sup>13</sup> that the stable fly (*Stomoxys calcitrans*) can take up the virus from the blood of infected monkeys and reinoculate it into healthy ones which will become paralysed. The experiment awaits confirmation and, after confirmation, convincing application to the circumstances surrounding infection in human cases of poliomyelitis.

<sup>13</sup> Rosenau, M. J.: Communication at the International Congress of Hygiene and Demography, Washington, 1912.

## DOMESTIC ANIMALS AS SOURCES OF INFECTION.

The frequent prevalence of epidemics in sparsely populated country districts has led, moreover, to consideration of domestic animals as sources of the infection.

Paralysis of dogs, horses, pigs, and fowl has been observed not uncommonly, but thus far without clear correlation with paralysis in man. Perhaps the most frequently observed coincidental paralytic diseases have been between hens and human beings. Undoubtedly since the wide prevalence of epidemic poliomyelitis, the existence of a paralytic disease among barnyard fowl has been more commonly noted. Possibly the condition has not actually become more frequent, but owing to the circumstance mentioned it has been oftener observed. It appears that the paralysis among fowl is caused, not by lesions of the central nervous system, but by lesions of the peripheral nerves, and is due to a peripheral neuritis.

It has not been found possible to transmit by direct inoculation the paralytic disease from chicken to chicken, or from chicken to monkey, or from paralytic monkey to chicken. However, it has been found possible to develop the paralysis in the laboratory by keeping the chickens in confinement for some time, and by supplying them an unusual and improper form of food. It has proved as little possible to transfer the paralytic affection of dogs from one individual to another by direct inoculation, or from dog to monkey, or from paralysed monkey to dog, or to set up paralysis in monkeys by inoculating them with nervous tissue obtained from paralysed pigs, or to produce paralysis in pigs with the virus of paralysed monkeys. These failures do not, of course, exclude the possibility that a reservoir for the virus may exist among domesticated animals that do not even respond to its presence by developing paralysis or other conditions which could be recognized as resembling poliomyelitis in man.

The manner of action of the virus of poliomyelitis in rabbits provides an illustration which shows how necessary it is to avoid general deductions in this field. At first it was strenuously denied that rabbits could be infected at all with the virus of poliomyelitis, and the examples of supposed successful inoculation reported were entirely disbelieved; but it must now be accepted that young rabbits

occasionally, but by no means generally, are subject to inoculation with the virus of poliomyelitis, at least after it has passed through a long series of monkeys.<sup>14</sup> Apparently a small percentage only of the inoculated rabbits develop any obvious symptoms, and these die, as a rule, during convulsive seizures which come on suddenly. A given virus has up to the present been sent through a series of six rabbits, after which it has failed to be further propagated. From the sixth series it has been reimplanted on the monkey, in which animal typical paralysis has been produced. It remains to add that the rabbits which succumb to the inoculation do not show any characteristic alterations of the central nervous system or other organs, as far as has been determined. The monkey, on the other hand, invariably shows the typical lesion of the central nervous system.

#### IDENTITY OF SPORADIC AND EPIDEMIC CASES.

Long before epidemic poliomyelitis had the wide distribution or claimed the attention now accorded it instances of infantile paralysis were known to everyone. Almost every community could point to one or more examples of the condition, and no one entertained the suspicion that the cause of the paralysis was an infectious or even contagious disease. Are these isolated cases of paralysis occurring among infants of the same nature as the epidemic paralysis, or has there merely been a confusion of names?

We possess means that permit an answer to this important question. Recovery, as you recall, is associated with enduring immunity, and the person or animal immune to poliomyelitis carries in his blood principles that neutralise the virus causing the disease. The blood of normal persons or animals lacks this property in any real degree. The test is, therefore, easily made: a mixture of the serum of the blood and virus is prepared, and after being in contact for a time is injected into a monkey. Thus it has been determined that the two diseases are caused by the same parasite, and it has been found that the neutralising principles are still present as long as 25 years after the attack of paralysis, and doubtless persist through life. This test has been employed likewise to identify

<sup>14</sup> Marks, H. K.: *Journal of Experimental Medicine*, 1911, vol. xiv., p. 116.

abortive cases of poliomyelitis in which paralysis has not appeared at all.

There is nothing unique in this apparently paradoxical situation. Most, if not all, of the epidemic diseases prevail at some time as sporadic affections—that is, as diseases of occasional occurrence. This is true of influenza, plague, and particularly of meningitis, with which poliomyelitis displays so many affinities. Knowledge is still very imperfect as to just what happens when an epidemic spread of a sporadic disease takes place. Sometimes conditions arise that favour rapid transference of the infecting microbe from individual to individual, through which a rise in virulence is accomplished very much as is done every day in the laboratory to enhance the potency of cultures. In respect to poliomyelitis, as seems also to be the case with meningitis, a fresh importation of an already enhanced virus probably occurs and is the immediate cause of the epidemic. The introduction may be at one point or at several points simultaneously, according to which the epidemic arises in, and spreads from a single centre or from many foci. Finally, sports or abnormally virulent parasites appear, prevail actively for a period, and then become reduced to an average degree of intensity, perhaps never to rise again. Some of the exceptionally severe epidemics of which history tells us may be thus accounted for. Such sports have been encountered in laboratories in regard to both pathogenic bacteria and protozoa.

#### QUESTION OF DIFFERENT STRAINS OF VIRUS.

Are biologically different strains of a poliomyelitic virus known? The evidence at hand is to the effect that different strains or races certainly exist if virulence be taken as the measure. German, Austrian, and French pathologists found that of the human specimens of spinal cord submitted to them for study about one-half could be inoculated successfully into monkeys, and less than this number could be propagated through successive animals. In America all the original specimens were successfully inoculated, but certain samples were far less active than others. At the beginning many of the inoculated monkeys survived the infection, sometimes with, sometimes without, enduring paralysis of leg or arm. Later

fewer survived, and after many passages of the virus from monkey to monkey, all became infected and all succumbed.

The Swedish virus of 1911 appears to be the most powerful yet studied. This is indicated by the fact that saline washings of the nose and throat and intestine could be inoculated successfully, after removal of all bacteria through filtration, in nearly every instance. In America it has been difficult to procure infection with these materials, from which it has been concluded that the virus displays degrees of infectiousness for monkeys. There are reasons for supposing that similar variations exist for man himself.

We may not, and probably shall not, know certainly whether this variability is restricted to the quality of virulence, or whether true types or races of the virus exist until artificial cultivation has been accomplished. Bacteriology has been singularly enriched recently by discoveries relating to biological types of certain microbes; and practical medicine is destined to benefit largely by the strong light which they have thrown upon perplexing questions of specific therapeutics. I am tempted to lead you aside a little way into this subject just because it is so full of suggestion and promise, and not merely with promise, since the fruits of discovery are being already tasted.

#### OBSERVATIONS ON PNEUMOCOCCI AND ANTIPNEUMOCOCCUS SERUM.

The pneumococcus causes many kinds of inflammation and one typical disease that prevails everywhere—namely, acute lobar pneumonia. Not infrequently there attend the pneumonia, and sometimes there appear independently, such inflammations as peritonitis, pleuritis, and meningitis, caused also by the pneumococcus.

Now pneumococci possess in common biological features regarded usually as sufficient to distinguish them—namely, form, staining properties, growth, virulence, and solubility in bile salts. But they have another quality that serves to distinguish them more finely, revealing different types among apparently similar organisms. By testing pneumococci from many different sources against an immune serum prepared with a single kind of the coccus it has been found that the cocci are not all alike, but that a predominant type



and several subsidiary types occur in nature.<sup>15</sup> Such a serum prepared with a given type of pneumococcus is neutralising alone for that one, and for no other one. The clinical reports on the anti-pneumococcus serum employed as a curative agent are contradictory, and one cause for this is now apparent.

Pneumococcus meningitis can be produced in monkeys by injecting subdurally by lumbar puncture a virulent culture of pneumococcus; it is invariably fatal. Antipneumococcus serum alone injected subdurally can change the outcome very little. But this infection is subject to combined chemo- and serum-therapy in which the chemical agent consists of sodium oleate that alone attacks and dissolves the pneumococcus. Acting separately, in the body, sodium oleate can accomplish little; it requires the assistance of the immunity principles. Acting together, the two agents quickly bring the infection under control and recovery follows. This happens even after the pneumococci have entered the blood stream and begun to multiply there. The effects of the soap and serum compound are, however, restricted to the type of pneumococcus represented by the immune serum in the mixture.<sup>16</sup> When the type of micro-organism and serum differ absolutely no therapeutic action follows. This obstacle to the practical employment of this method of specific treatment will doubtless be reduced or even wholly set aside by preparing a true polyvalent immune serum that will represent not many cultures of the pneumococcus taken at random, but the several types or races occurring in nature. We already know the number to be few.

It has become the custom to speak of these types of microbes as resistant or "fast," but the term is relative merely. The fact and degree of fastness will be revealed by the source of the test-serum. But within a given microbic species this quality of resistance may well appear against chemical bodies as well. Pneumococci, for example, vary in properties by gradual gradations in the direction of

<sup>15</sup> Neufeld, F., and Händel: *Zeitschrift für Immunitätsforschung, Originale*, 1909, Band iii., p. 159; *Arbeiten aus dem kaiserlichen Gesundheitsamte*, 1910, Band xxxiv., p. 293; *Berliner klinische Wochenschrift*, 1912, Band xlix., p. 480.

<sup>16</sup> Lamar, R. V.: *Journal of Experimental Medicine*, 1911, vol. xiii., p. 1; 1912, vol. xvi., p. 581.

the streptococcus, which, besides differing in still other biological properties, chances not to dissolve in bile. The gradients of pneumococci approaching the streptococcus are progressively less acted upon by sodium oleate. The trypanosome of sleeping sickness is less subject to the therapeutic action of certain organic arsenic compounds in some regions in Africa than in others. The antimeningitis serum suppresses the growth and multiplication of most meningococci, but not of all. This quality of fastness is not alone innate, but can be developed artificially as a mutation both against serum principles and chemical drugs and may persist.

Infectious diseases showing a strong tendency to relapse in course of recovery are caused by microbes tending to flourish as races or types. Relapsing fevers that pass through three or four exacerbations on the way to recovery are attributed to spirochætæ assuming a corresponding number of distinct forms. Infections tending to many relapses, of which lues is an example, are attributed to parasites capable of flourishing in many such types of which one part is innate and the other the result of mutations under the influence of curative serum or drug. Fortunately, there appears to be no parasite capable of performing indefinite mutations; and experience is teaching that the more precise, specific, and vigorous the means employed to control infection, the smaller the risk of mutation and the greater the probability of suppression of the parasitic agent of disease.

In 1886<sup>17</sup> Theobald Smith first clearly pointed out that the injection of dead bacteria confers active immunity to subsequent inoculation with virulent materials. Now the employment of dead bacteria is widespread, both for preventing and for healing disease. Wright especially is to be credited with the general application of the method to therapeutics. While the limits of value of inoculation, as it is termed, are not yet defined and it promises, theoretically, more for the subacute and chronic than for the acute infections, I am inclined to the belief that to be really effective attention will need more and more to be accorded to the question of specific type in the infecting bacteria.

<sup>17</sup> Salmon, D. E., and Smith, Theobald: Proceedings of the Biological Society of Washington, 1884-6, vol. iii., p. 29.

## DEFICIENCY OF CEREBRO-SPINAL FLUID IN CURATIVE PRINCIPLES.

In pursuing the devious courses of infection, of which examples have just been given, the fact has emerged that the effectiveness of curative means will be determined not only by the intrinsic qualities of the parasites, but also in a high degree by the manner of location and distribution of the parasites themselves within the infected host. Whether they have a general distribution throughout the blood and tissues, or whether they are confined within an important organ or part, may be the factor determining the ease with which they can be reached, not only by the natural curative principles of the body, but also by artificial curative agents introduced into the body.<sup>18</sup>

The parasite, struggling to survive, withdraws, at one time, into situations to which the curative substances gain access imperfectly and with difficulty, causing thereby local infections more or less cut off from the general circulation and the curative substances purveyed by the blood. This is the condition met with in localised inflammation and in infections of specialised portions of the body, such as the great serous cavities, that receive a modified and dilute lymph secretion carrying reduced quantities of the protective principles contained within the blood. The quality of lymph in the several serous cavities and in the various tissues is not the same, and the lowest limit of strength is reached by the cerebro-spinal fluid that functions as the lymph of the brain and spinal cord. The exclusion of dissolved substances from the cerebro-spinal liquid is a provision of great importance, but is not an unmixed good. For while it affords protection to the sensitive nervous tissues from injurious chemicals, it deprives them also of curative principles.

Happily this deficiency has now been superseded by a method of direct local treatment by injections that has given excellent results in meningitis, and is now being employed in luetic affections of the meninges and central nervous organs with encouraging results.<sup>19</sup>

<sup>18</sup> Flexner: Boston Medical and Surgical Journal, 1911, vol. clxv., p. 709; the Harben Lectures, Journal of State Medicine, 1912, Nos. 3, 4, and 5.

<sup>19</sup> Swift and Ellis: New York Medical Journal, 1912, vol. xcvi., p. 53. Wechselmann: Deutsche medizinische Wochenschrift, 1912, Band xxxviii., p. 1446.

## TREATMENT.

Remote as some of them may seem, the considerations to which I have called your attention have a bearing more or less vital upon the problem of a specific and effective treatment of poliomyelitis.

Poliomyelitis is not a disease with a very high mortality; its chief terror lies in its appalling power to produce deformities. When death does occur it is not the result, as in many infections, of a process of poisoning that robs the patient of strength and consciousness before its imminence, but is caused solely by paralysis of the respiratory function, sometimes with merciful suddenness, but often with painful slowness, without in any degree obscuring the consciousness of the suffocating victim until just before the end is reached. No more terrible tragedy can be witnessed.

I have already laid before you certain facts regarding immunity in poliomyelitis, and it remains to be added that the employment for treatment of the immune serum, taken from monkeys or from human beings, exercises a definite if not very strong protective action upon inoculated monkeys. Either the disease is prevented altogether or its evolution is modified in such a manner as to diminish its severity. When the virus used for inoculation is highly adapted to the monkey, and thus very virulent, it is more difficult to control the result than when it departs less from the original human type and is less active.

The immune serum has thus far acted best when it was injected into the subdural space on several successive days. This is in conformity with the fact that, however introduced into the body, the virus establishes itself in communication with the cerebro-spinal liquid, where it propagates for a time. After a time the virus localises in the nervous tissue itself, and becomes accessible not only from this liquid, but probably from the general blood also. The serum introduced into the subdural space soon escapes into the blood; and thus a double action is secured. On the one hand it reaches the nervous tissue directly from the cerebro-spinal liquid, and on the other with the blood. An immune horse serum at first gave disappointing results, but latterly its employment by intramuscular injection has given more promise. But none of the sera

mentioned can be regarded as having more than touched the fringe of the problem of a cure for the disease.

Such brilliant success has been recently recorded in respect to the specific chemical therapeutics of infection that an effort has been, and still is being, made to attack the problem from this quarter. Here, also, only a starting point has been secured and the subject merely opened to further experimentation. The point of departure which we have adopted is the drug hexamethylenamine (urotropine), which possesses a degree of antiseptic action in the body and is known to be secreted into the cerebro-spinal liquid. When the drug is administered by mouth it can be detected by chemical tests in the liquid in a short time. When inoculation of virus and administration of the drug are begun together and the administration continued for some days afterwards, the development of the paralysis is sometimes but not always averted.

Hexamethylenamine lends itself to modifications by the addition of still other antiseptic groups to its molecule. We have tested a large number of such modifications and have found certain ones to exceed the original compound in protective power, and others to promote the onset of paralysis. This is the common story of drugs. None are wholly without some degree of injurious action upon the sensitive and vital organs of the body. But manipulative skill has already succeeded in eliminating the objectionable and improving the valuable features of drugs so that they exert their action but little upon the organs and severely upon the parasites when they become useful as therapeutic agents. This process may be called sundering the organotropic and parasitotropic effects. Whether this can be successfully accomplished with this class of compounds cannot be predicted. But if not the quest will be transferred to still other drugs. When it is accomplished the victory will be won. By whom will the victory be won, and when? Ours is the office of story-teller and not the vision of the prophet!

In giving Huxley to science the Charing Cross School of Medicine conferred a great benefit upon the world. In imbuing him with the ideals of biological science it performed an especial service for America. For in 1876 Huxley journeyed to Baltimore to deliver

the address at the formal opening of the Johns Hopkins University, at which time he outlined in essence the plan of medical education which, 20 years later, was adopted and put into practice at the Johns Hopkins Medical School. The example of this wise foundation, inspired by Huxley, has acted far and wide throughout the United States as a regenerating force upon medical education.

## A FILTERABLE AGENT THE CAUSE OF A SECOND CHICKEN-TUMOR, AN OSTEOCHONDROSARCOMA.

By PEYTON ROUS, M.D., JAMES B. MURPHY, M.D.,  
AND  
W. H. TYTLER, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)*

The demonstration of the cause of a transplantable spindle-celled chicken-sarcoma<sup>1</sup> led us last winter to a further study of tumors of the fowl. Spontaneous chicken-tumors are far from rare, and several excellent descriptions of them exist.<sup>2</sup> In the course of ten months we obtained without difficulty about thirty various tumors in the living fowl. None of the growths was quite similar histologically to the sarcoma just mentioned, which is caused by a filterable agent, but there were two that, like it, proved transplantable,<sup>3</sup> namely, a tumor producing true cartilage and bone (Chicken-Tumor 7) and a curious spindle-celled sarcoma (Chicken-Tumor 18). We wish here to report experiments showing that the osteochondromatous tumor is caused by a filterable agent.

The original osteochondroma appeared as a discrete mass on the breast-bone of an adult fowl. It was very hard, appeared to be of long standing, and projected symmetrically to either side of the sternal keel. Microscopically as well as clinically it seemed benign. That it grew on transplantation was surprising, but the less so since several mammalian growths of character apparently benign have proved transplantable, notably a chondroma of the mouse (Ehrlich) and a fibroma of the dog (Ribbert). Murray<sup>4</sup> has described a "chondro-osteoidsarcoma" of the mouse which retained on trans-

1. Rous, Peyton: THE JOURNAL A. M. A., Jan. 21, 1911, p. 198.

2. Ehrenreich and Michaelis, Tyzzer, Wernicke.

3. Rous, Murphy and Tytler: THE JOURNAL A. M. A., June 1, 1912, p. 1682.

4. Murray, J. A.: Third Scientific Report of the Imperial Cancer Research Fund, 1908, p. 78.

plantation its ability to produce bone and cartilage. In the case of the chicken-tumor the growths derived from transplantation consist, like the original tumor, of large cells of connective tissue about which cartilage is soon laid down, followed, in many instances, by true bone containing red marrow. The neoplasm is now in its eighth transplantation generation. In some instances its growth is continuous, though often very slow, and as the mass of cartilage and bone becomes large the host emaciates and finally dies. In other instances the tumor sooner or later becomes stationary and may so remain for months. Recently metastases showing cartilage have been noted in the case of two of the most rapidly growing tumors. There is now prominent in the growth a spindle-celled element and it must be regarded as an osteochondrosarcoma. The general characters of the tumor are described in full in a paper by Dr. Tytler forthcoming in the *Journal of Experimental Medicine*.

Our attempts to determine a cause for the osteochondrosarcoma were not begun until after it had been several months under observation, and in numerous hosts. During this time its general character had altered little. The sarcomatous element was not noticeable and the growth appeared benign. Differential filtration was the method used. Sound portions of the tumor tissue were ground with sterile sand and taken up in a considerable bulk of Ringer's solution; shaking was done in a machine for from twenty to thirty minutes; centrifugalization followed; and then the supernatant fluid was passed through a filter of fine texture (Berkefeld) that held back all tumor-cells. A few cubic centimeters of the clear filtrate were injected into the trunk or leg muscles of a number of normal fowls. Sterile infusorial earth was in many instances added to the filtrate previous to its injection, since the tissue derangement caused by this foreign body has been found to favor the action of the agent engendering Chicken-Tumor 1,<sup>5</sup> the spindle-celled sarcoma.

For a first experiment a large Berkefeld filter (No. 8, V) was employed, without bacterial control as to its permeability. Of the ten fowls inoculated with 10 c.c. of the filtrate in each breast, one had developed thirty-eight days later a mass 0.3 cm. in diameter in the muscle at one of the inoculation sites. At the end of another

5. Rous, Murphy and Tytler: THE JOURNAL A. M. A., June 8, 1912, p. 1751.



month, when the fowl died of intercurrent causes, this nodule measured 0.5 cm. It was very hard and consisted of the characteristic, cartilaginous tumor tissue, which, in this instance, contained no bone. At the present date, four months after the injection, the remaining fowls have not developed tumors.

In the later work Berkefeld filters of demonstrated impermeability to *Bacillus fluorescens liquefaciens* have been used. The development of malignancy in the chondroma, as shown by the production of metastases, has furnished a material which *a priori* would seem more suitable for the work. That it is indeed more suitable has been proved by the prompt development of swiftly growing tumors after the injection of the filtrate. For the experiment that follows there was taken the metastatic ovarian tumor of a fowl which carried in both pectoral regions large bony tumors as the result of implantation, and had in the ovary two hemorrhagic, cartilaginous metastases, each nearly as large as a hen's egg, together with diffuse implantation growths of the cartilage-forming connective tissue over visceral and parietal peritoneum.

#### EXPERIMENT.

Cartilaginous tumor tissue to the amount of 11 gm. from the ovarian metastases of Fowl 352, sixth Generation A, was ground in a mortar with sterile sand. The resulting mass was taken up in about 350 c.c. of Ringer's solution warmed to body heat, and the whole was shaken in a machine for twenty minutes and briefly centrifugalized to sediment the tissue fragments. The cloudy supernatant fluid was passed through a Berkefeld filter V, size No. 3, which was tested both before and after the experiment under conditions similar to those obtaining in it, and was found to hold back *B. fluorescens liquefaciens* out of a suspension obtained by shaking. The tumor extract passed readily through the filter. The fluid thus procured was limpid and reddish-yellow (owing to hemorrhages into the cartilage).

Two portions of the filtrate, one with, and one without a little sterile infusorial earth (*Kieselguhr*), were injected into each of six normal fowls of the breed in which the spontaneous tumor was found (Plymouth Rock). Of the filtrate plus *Kieselguhr* 4 c.c. were used for each injection; of the filtrate alone 6 c.c. The pectoral and thigh muscles were employed as sites.

Eighteen days later the first examination was made. In four of the fowls tumors were found where filtrate plus *Kieselguhr* had been injected. The growths measured from 1 to 3 cm. across and were evidently due to the coalescence of numerous smaller nodules. In two of the four fowls a small, discrete nodule was palpable where the filtrate alone had been injected. It lay in the track of the injecting needle. All the growths were very hard. One of these fowls was at once killed and the growth looked at. Macroscopically cartilage could be seen in it. Microscopically it consisted of cartilage and a tissue, com-

posed of numerous spindle-shaped or stellate cells, which was undergoing a cartilaginous change.

Twenty-five days after the injection the tumors were even harder than before and were growing rapidly. One now measured 4.7 cm. across. In two of the fowls the findings were still negative.

#### SIGNIFICANCE OF THE FINDINGS.

The experiments show that the osteochondrosarcoma, like our spindle-celled sarcoma, can be produced in fowls hitherto normal, by an agent separable from the tumor cells and capable of passing through a Berkefeld filter which holds back *B. fluorescens liquefaciens*. Furthermore, the action of the agent appears to be dependent, like that producing the sarcoma, on a cell-derangement brought about by accessory factors. The addition to the filtrate of sterile infusorial earth to bring about a reactive tissue proliferation resulted in a high percentage of successful inoculations, each tumor arising from numerous local points; whereas the filtrate when injected alone in the same fowls caused a tumor much less frequently and then as a single discrete nodule in the track of injury from the injecting needle.

Despite these points of similarity the agents causing the chicken sarcoma and chondroma can hardly be identical. They give rise to widely different tumor forms. Previous to the experiments here related we had supposed it possible that a single filterable agent by its localization in cells of different potentiality might give rise to different tumors; to test the point we had made numerous attempts to obtain a localization of the agent causing Chicken-Tumor 1 in other cells than those which it usually influences. These attempts were all unsuccessful. And now it appears that the histologic character of the osteochondrosarcoma is due to a peculiarity of the causative agent which is retained when the latter is separated from the tissue of the growth. Thus the agent, when brought into contact with the connective tissue in voluntary muscle, produces not an ordinary spindle-celled sarcoma, but a growth that elaborates cartilage and finally bone. That an extracellular agent should cause a reversion of connective tissue to the so-called embryonic type would not appear strange from what is already known of the changes that connective tissue undergoes in wounds, or when cultivated in

*vitro*; but that such an agent should bring about a differentiation ordinarily foreign to the tissue is very remarkable. Yet in this connection it should not be forgotten that bone and, much more rarely, cartilage are sometimes laid down in connective tissue under relatively simple pathologic conditions.

The nature of the agent causing the osteochondrosarcoma cannot at present be stated. The agent causing the spindle-celled sarcoma is probably a living virus.<sup>5</sup> The demonstration that extrinsic agents are the cause of two connective-tissue growths of the fowl which are characteristic malignant tumors renders it necessary to suppose either that such tumors of the fowl have an entirely different etiology from mammalian tumors, or else that the latter are of similar origin. In any case the findings with the chicken-tumors largely demolish the theoretical basis on which objections to an extrinsic cause for cancer have been built up.

## IMMUNIZATION BY MEANS OF CULTURES OF TRYPANOSOMA LEWISI.\*†

By F. G. NOVY, W. A. PERKINS, AND R. CHAMBERS.

(From the Hygienic Laboratory, University of Michigan, Ann Arbor.)

In a previous paper<sup>1</sup> it was shown that cultures of *Tr. lewisi* when plasmolyzed in collodium sacs, against distilled water, underwent changes resulting apparently in complete solution or rather disintegration of the organisms. Three or more injections, on alternate days, of such plasmolyzed cultures protected young rats against a minimal infective dose of virulent blood. Immunity could therefore be produced by the injection of disintegrated cultural trypanosomes.

At the time it was also pointed out that cultures of *Tr. lewisi* and *Tr. brucei*, even after they had passed through a hundred generations or subcultures, in the course of two years, do not become attenuated by such prolonged consecutive passage but readily infect. This statement is only partially true, since the organisms on prolonged cultivation actually do suffer some loss of virulence or capacity to infect. A culture of *Tr. brucei* in the 100th generation will infect rats but the period of incubation is somewhat lengthened, and while death may occur within two or three weeks, it may not result until several months have elapsed from the time of inoculation; the longest survival is one which is at present in its 253d day. Obviously, the conditions which obtain in cultivation, notably the composition and reaction of the medium, the temperature and duration or age of culture, are not without appreciable effect on these organisms.

From the beginning, when the cultivation of *Tr. lewisi* and *Tr. brucei* was first effected, we have looked forward to the production of attenuated strains which could be utilized in preventive inocula-

\* Received for publication August 24, 1912.

† This research has been aided by a grant from The Rockefeller Institute for Medical Research.

<sup>1</sup> *Proc. Soc. Exp. Biol. and Med.*, 1907, 4, p. 42.

tions. It seemed reasonable to believe that the cultures of pathogenic protozoa, like those of bacteria, would be found available to some extent in the production of immunity. The fact that spontaneous recovery may occur in protozoal diseases, as is the case with relatively insusceptible species, indicates the establishment of an acquired immunity, and hence the inherent capacity possessed by such an animal to protect itself against the given parasite. The trypanosomal infection of the common rat is a most excellent illustration of this fact, since it only exceptionally proves fatal. In the course of some weeks, or months, the rat gets rid of the trypanosome and becomes immune to a subsequent inoculation. It is evident, therefore, that the cultures of this parasite are particularly suitable for work bearing upon trypanosomal attenuation and immunity. With this organism it has been possible to demonstrate two important facts, first, that attenuation can be secured, and second, that such living, attenuated cultures confer a solid and permanent immunity.

The culture of *Tr. lewisi* which we have used was isolated from a rat September 5, 1905, since which time it has been maintained on ordinary blood agar medium, at 25°. It is at present in the 313th generation. At first, the culture was readily infective; the growth from a single tube being sufficient to produce typical infection in the rat. This infective property persisted for a considerable period and was frequently utilized to supply infected rats for the laboratory. After a long interval, in March, 1909, this procedure to secure a rat infection was again resorted to, the culture then being in the 158th generation. Contrary to expectation, no infection resulted. During the next few weeks no less than 17 rats were tested by intraperitoneal injections, each receiving from two to four cultures. In every case, as before, the result was negative, although daily examinations were made for an entire month. These tests clearly demonstrated that this stock culture of *Tr. lewisi* had completely lost its capacity to develop within the rat. In other words, it had become fully attenuated.

The many inoculations made with this culture during the past three years have invariably failed to produce infection. The result is the same whether the contents of a single tube or of 50 tubes are

injected into the rat. Such cultures, grown at 25° for seven to 10 days, were extremely rich in flagellates which at times formed a thick slimy covering on the blood agar medium. Hence, the quantity of flagellates injected was at times enormous, and yet, notwithstanding this fact, no infection could be produced. The microscopic appearance of the cultures was in no wise different from that of the early generations which gave positive results, but the parasitic property of the organism was definitely lost.

All attempts to restore the infectious character to the culture have been fruitless. Even when the culture was placed in collodium sacs, in the peritoneal cavity of rats, it failed to multiply. Possibly further efforts in this direction may be successful.

While it is true, as has been emphasized above, that the cultures are no longer infective, it is an interesting fact that the majority of the inoculated rats do show in their blood, within the next day or two, a small number of fairly typical blood trypanosomes. These, however, do not increase in numbers and usually disappear within 48 hours after the injection. After that they are not to be found even though most careful examinations are made for a month or more. With the hope of restoring the virulence, the blood of rats showing these early trypanosomes was drawn from the heart and injected<sup>1</sup> into clean young rats and was also planted at the same time on blood agar. Much to our surprise, it was found that such blood failed to infect the rats and also failed to give cultures in the tubes.

The failure to secure a growth on the blood agar medium was particularly surprising, since, as is well known, this cultural method is capable of revealing trypanosomes even when prolonged microscopic examinations are negative. In all, about 12 rats which showed these transitory trypanosomes were cultured without obtaining the slightest indication of a growth. Similarly, the inoculation of nine rats with such blood, the doses varying from 0.25 c.c. to 3 c.c., failed to infect. Here, as above, the negative result could not be ascribed to the small number of trypanosomes, for it can be readily shown that the normal trypanosomal blood will infect, when diluted, to such an extent that the parasites can scarcely be detected under the microscope. Likewise, the susceptibility of our rats could

<sup>1</sup> All injections unless otherwise indicated were given intraperitoneally.

not be called into question. In all of our work special care was taken to use only young rats, weighing from 60 to 90 gms., which had been repeatedly examined for two or more weeks and thus found to be free from natural infection. When used as controls, the injection of 1 c.c. of a 1:10,000 dilution of blood containing *Tr. lewisi* invariably gave a rich infection.

It is evident, therefore, that the trypanosomes which appear transiently in the blood of rats after the injection of a culture are incapable of further multiplication or reproduction. They are sterile or abortive forms, and without doubt their further study will prove of interest. It would seem as if some of the cultural trypanosomes still possessed a tendency to develop into the blood form, but the resulting individual through lack of inherent vigor or failure of fertilization (?) is unable to develop further and soon disappears.

It is probable that these abortive forms are present in the blood of all rats inoculated with the cultures. They are, however, easily overlooked and for that reason they have been found in only 75 per cent of the rats examined. They have been observed in the blood on one occasion five hours after the injection; at other times within 12 hours, but more often between 24 and 48 hours. Only exceptionally have they been seen as late as 72 hours and once they were observed to be present for four days. As already stated, the abortive forms are to be found in small numbers only. At times, after a prolonged search, but a single one can be found; more often, one can be found for every five or ten fields (No. 7 objective); and, rarely, one for every two fields examined. The number of abortive forms is independent of the dose injected, for in rats which received 50 cultures they were no more numerous than when a single culture was given. Likewise, the age of the culture seems to have no influence upon the number of these forms.

#### IMMUNITY.

As already stated, our previous studies have shown that cultures of *Tr. lewisi* when plasmolyzed<sup>1</sup> were capable of producing immunity

<sup>1</sup> This method of preparing a vaccine, though not generally applicable, has been utilized in this laboratory by Dr. J. G. Cumming, in charge of the University Pasteur Institute, in the preparation of a safe antirabic vaccine. His experiments have shown that the dialyzed virus protects animals even more efficiently than does the desiccated virus prepared in the ordinary way.

in rats. It was therefore to be expected that the living attenuated cultures would behave in a similar way and confer protection against the blood trypanosome. The experimental work in this direction demonstrated that such was the case and that an active immunity was developed in animals which had received single or multiple injections of the living cultures.

In order to obtain absolutely conclusive results it was necessary to employ young rats, preferably those raised in the laboratory, which repeated examinations for at least two weeks before use had shown to be free from the natural infection. In the beginning, when rats which had been purchased on the market were used, it not infrequently happened that the control animals in an experiment would fail to become infected, thus invalidating the immunity test. This difficulty was entirely overcome by employing only carefully tested young rats.

Another point of importance, not infrequently lost sight of in immunity tests, is the use of a very small but surely infective dose of the virulent organism, for the reason that a given degree of immunity may be effective against a minimal infecting dose but not against a hundred or thousand doses. A very large dose of virus may readily break down a slight immunity and thus lead to the erroneous conclusion that no protection exists. With this consideration in mind, we have as a rule employed the test virus in a dilution (in 0.85 per cent salt solution) of 1:10,000. One cubic centimeter of this suspension has never failed to produce a rich infection in clean young rats. In some instances, as shown in the tables, suspensions of 1:200 or 1:2,000 were used.

On examination of Table 1, several interesting facts will be noted. When the interval between the injection of the vaccine and the test dose is but two or three days (rats Nos. 3, 20, 21, 9, 27) a rich infection results, showing that little or no immunity exists at that time. Possibly a smaller dose of the test virus might have shown some immunity at this early date, because this is certainly evident on the fourth day (Nos. 5 and 23). On the other hand, but one of the two rats inoculated on the eighth day (No. 28 which received six cultures) developed sufficient resistance to abort the infection.



TABLE I.  
*Immunity Tests Made after a Single Injection of*

2 CULTURES					6 CULTURES				
Rat No.	Interval between Injections	Test Dose of <i>Tr. lewisi</i> Blood	Result	Remarks	Rat No.	Interval between Injections	Test Dose of <i>Tr. lewisi</i> Blood	Result	Remarks
1....	14	0.005 C.C.	⊕	1 trypanosome on third day	18....	14	0.005 C.C.	0	
2....	14	"	0		19....	14	"	0	Rich infection
3....	2	0.0005	+	Rich infection	20....	2	"	+	"
4....	18	"	0		21....	2	0.0005	+	
5....	4	0.0001	0		22....	18	"	0	
6....	9	"	0		23....	4	0.0001	⊕	Very mild infection; 1/10 per field for three days
7....	13	"	0						
8....	16	"	⊕	Very mild infection; 1 per field for seven days	24....	9	"	0	
9....	3	"	+	Rich infection	25....	13	"	0	
10....	8	"	+	"	26....	16	"	0	
11....	12	"	0		27....	3	"	+	Rich infection
12....	16	"	0		28....	8	"	⊕	Very mild infection; 1 per field for six days
13....	10	"	0						
14....	10	"	⊕	Very mild infection, 1 per field for nine days	29....	17	"	0	
					30....	17	"	0	
15....	10	"	0		31....	17	"	0	
16....	10	"	0		32....	17	"	0	
17....	10	"	0		33....	17	"	⊕	Very mild infection; 1-5 per field for 4 days

In the tests tabulated above, controls were inoculated each time after the experimental animals, and developed a rich and lasting infection.

0 indicates no infection, i.e., immunity is present.

⊕ indicates a rich infection, the same as that in the controls.

⊕ indicates a mild or abortive infection characterized by the presence of very few trypanosomes per field in fresh blood preparation.

Very mild or abortive infections may occur, as will be seen, in rats inoculated even on the 10th (No. 14), 14th (No. 1), and 17th (No. 33) day, but in these the parasites were, as a rule, very scanty and persisted for but a few days. These infections correspond to those which can be produced by injecting a large dose of trypanosomal blood into rats which have recovered from a natural infection. The breaking down of the immunity in such cases is usually indicated by a mild infection of but a few days' duration.

Altogether it will be evident that immunity is produced by the injection of living cultures of *Tr. lewisi* and that this immunity is most pronounced after the lapse of about 10 days. In the experiments discussed, the smallest dose of vaccine was represented by the growth present in two culture tubes.<sup>1</sup> The minimum dose of the living culture, sufficient to immunize, is probably a very small fraction of that mentioned. Thus, in one experiment, four rats were inoculated with the growth corresponding to one-fourth, one-half, one, and two culture tubes, respectively. After an interval of 12 days, these rats and two controls were inoculated with 0.0001 c.c. of trypanosomal blood. The treated rats showed no infection, whereas the controls became heavily infected.

*Subcutaneous Injection of Cultures.*—As might be expected a notable difference in immunity production is obtained according to whether the vaccine is introduced intraperitoneally or subcutaneously. When introduced by the latter route a much larger dose of the culture must be given in order to obtain protection. Only one experiment with single injections, given subcutaneously, was made and is worthy of note. As shown in Table 2, the rats which received two cultures became infected as heavily as did the controls; whereas those which received six cultures at the same time showed a mild abortive infection which persisted for one and three days, respectively. Evidently a single injection given subcutaneously is much less effective than when given intraperitoneally, for while a fraction of a culture is sufficient when administered by the latter

<sup>1</sup> The tubes used for cultures were small, being but 12 × 150 mm. After inoculation the tubes were placed in a hot room at 25° for from seven to 12 days. The growth was then taken up in 0.85 per cent salt solution and at once injected.

It may be stated in this connection that the injection of an extract from uninoculated tubes has no immunizing action.

route, a much larger dose is necessary when injected under the skin.

When several injections are given subcutaneously, the resulting immunity is much more marked, since the absorption of the antigen is necessarily greater. One experiment of this kind is given in Table 2 and shows perfect protection.

TABLE 2.  
*Immunity Tests after Subcutaneous Injection of Living Cultures.*

Rat No.	Received	Interval before Test Dose	Test Dose of <i>Tr. lewisi</i> Blood	Result	Remarks
1.....	2 cultures	18 days	0.0001 c.c.	+	Rich infection
2.....	" "	" "	" "	+	" "
3.....	6 cultures	" "	" "	⊕	Mild infection lasting 3 days
4.....	" "	" "	" "	⊕	Mild infection lasting 1 day
					Two controls gave a rich infection
5.....	5 injections in 5 days each of 2 cultures	17 days	" "	0	Perfect immunity
6.....	5 injections in 5 days each of 2 cultures	" "	" "	0	" "
7.....	5 injections in 5 days each of 2 cultures	" "	" "	0	" "
					Four controls gave a rich infection

*Multiple Injections.*—In Table 2, Nos. 5, 6, and 7 show that multiple injections of the culture given subcutaneously impart a marked degree of immunity. A number of similar tests were made, the injections being intraperitoneal, in order to obtain hyperimmunity and to test the resistance of such rats to relatively large doses of trypanosomal blood. The rats thus immunized, while fully protected against a minimal dose of virus, on the injection of a fairly large dose, such as 0.25 c.c., may show a mild abortive infection which disappears in from two to three days. An exactly similar "break down" of immunity frequently occurs when a large dose of infective blood is injected into rats which have spontaneously recovered from an infection with *Tr. lewisi*. These abortive infections depend largely upon the dose and to some extent upon the rat itself.

The result of these tests is given in Table 3. By way of explanation it should be stated that Nos. 1–4 received in the course of 12

days three injections each of six cultures, Nos. 5-7, in 58 days, received three injections, each of two cultures, while Nos. 8-10 in the same time were also given three doses, each of four cultures and Nos. 11-13 received similar injections, each of six cultures. Nos. 14-21 were given in the course of 67 days six injections, the first four receiving two cultures each time and the last four, six cultures.

TABLE 3.  
*Immunity after Multiple Injections.*

Rat No.	Received	Interval before Test Dose	Test Dose of <i>Tr. lewisi</i> Blood	Result	Remarks
1	3×6 C 12 days	13 days	0.25 c.c.	⊕	Tr. present for two days
2....	" " " "	" "	" "	⊕	" " " three "
3....	" " " "	" "	" "	o	Full protection
4....	" " " "	" "	" "	⊕	Tr. present for two days
					Four controls gave rich infection
5....	3×2 C 58 days	5 days	0.1 c.c.	o	
6....	" " " "	" "	" "	o	
7....	" " " "	" "	" "	⊕	Tr. present for four days
8....	3×4 C " "	" "	" "	o	
9....	" " " "	" "	" "	o	
10....	" " " "	" "	" "	o	
11....	3×6 C " "	" "	" "	o	
12....	" " " "	" "	" "	o	
13....	" " " "	" "	" "	o	
					Two controls for this set gave rich infection
14....	6×2 C 67 days	7 days	0.005 c.c.	o	This set of eight were injected two weeks later with 0.25 c.c. of virulent blood. All resisted perfectly this second injection
15....	" " " "	" "	" "	o	
16....	" " " "	" "	" "	o	
17....	" " " "	" "	" "	o	
18....	6×6 C " "	" "	" "	o	
19....	" " " "	" "	" "	o	
20....	" " " "	" "	" "	o	
21....	" " " "	" "	" "	o	
					Four controls for this set gave rich infection

#### DURATION OF IMMUNITY.

Since a single injection of the living culture protects against a surely infective dose of *Tr. lewisi*, it was of interest to ascertain the duration of such immunity. This active immunity becomes manifest in about 10 days and once developed it probably persists until the death of the animal. Up to the present time we have not tested out the immunity for a longer period than five months, but there can

be little doubt that it will be found to last for a considerably longer period.

In Table 4, which may be looked upon as a continuation of Table 1, are brought together the several tests in which the interval between the injections exceeds 18 days. The several groups were tested at different times with the usual dose of 0.0001 c.c. of trypanosomal blood. In every case controls were inoculated at the same time and invariably gave a rich infection.

TABLE 4.

*Duration of Immunity Following the Single Injection of Living Cultures.*

Rat No.	Received	Interval before Test Dose	Test Dose of <i>Tr. lewisi</i> Blood	Result	Remarks
1....	2 cultures	35 days	0.0001 c.c.	0	A single trypanosome was found on each of two days
2....	" "	" "	" "	0	
3....	6 "	42 "	" "	0	
4....	" "	" "	" "	0	
5....	6 "	74 "	" "	0	
6....	" "	" "	" "	0	
7....	2 "	78 "	" "	0	
8....	" "	" "	" "	⊕	
9....	" "	84 "	" "	0	
10....	" "	" "	" "	0	
11....	" "	112 "	" "	0	
12....	6 "	119 "	" "	0	
13....	2 "	150 "	" "	0	
14....	6 "	159 "	" "	0	

#### IMMUNIZATION AGAINST NATURAL INFECTION.

The marked protection following the introduction of living cultures suggested the possibility that vaccinated rats would be equally protected against natural infection by the flea or louse. The number of parasites present in the test dose of blood employed in the previous experiments must be considerably larger than can be introduced through the agency of a biting insect. If, therefore, no other factor is involved, it should be possible to protect the rats against the natural infection by means of insects..

The white rat which we have used exclusively has never been found in this laboratory to harbor fleas and consequently we have been unable to undertake experiments with this insect. The rats, however, are often heavily infected with lice, and since, unquestion-

ably, these as well as fleas are agents of transmission, we endeavored to protect the rats against this source of infection.

In our first experiment we tried to secure infection by the direct transfer of lice from infected rats to a set consisting of four clean and four vaccinated rats, each of the latter having previously been given an injection of six cultures. Although considerably over 100 lice were transferred to each rat, no infection followed. Later on, therefore, these rats were confined in a small space with infected rats which were heavily infested with lice but the result was no better. At the end of five months, although blood examinations were made twice weekly for over four months, no trypanosomes could be detected in the exposed rats. The failure of the controls to become infected was not due to an unrecognized infection, since one of these inoculated on the 133d day with 0.0001 c.c. of virulent blood gave a typical infection.

For the second experiment 10 rats were injected each with six cultures. Five of these (see Table 1, Nos. 29-33) were tested 17 days later and found to be immune. The remaining five, together with five clean rats for control, and five rats containing *Tr. lewisi* and heavily infested with lice, were placed in a small cage where they remained for several months. The rats were examined at least once a week for two months but no spread of the infection occurred.

A third experiment was more successful. As above, 10 rats were first injected each with two cultures and after 10 days, five of these were tested and found to be immune (see Table 1, Nos. 13-17). The remainder, together with five clean and eight lousy, infected rats, were confined in a small cage. Later five additional infected and lousy rats were added. Of the vaccinated rats four died on the 24th, 29th, 52d, and 130th day, respectively, without at any time showing trypanosomes in their blood. Of the five clean controls, two died on the 61st and 106th day without becoming infected. The remaining three became infected on the 28th, 69th, and 81st day. The fact that three out of five non-immunes and none of the vaccinated animals became infected would indicate that the living vaccine does protect against infection by the louse. Further tests, however, especially with directly transferred lice, will be necessary fully to establish this point. It seems from the work done thus far

that the lice from young rats which have been inoculated with *Tr. lewisi* but a few days before are more likely to convey the infection than those from rats having an old chronic infection.

#### CURATIVE ACTION OF CULTURES.

The readiness with which the living culture immunizes against infection opens the possibility of using such material to abort, or in other words, cure an infection already under way. The extremely rapid and heavy infection in the case of *Tr. lewisi* makes this at the outset a very unpromising attempt; for when a relatively small dose of trypanosomal blood, as shown in Table 3, may serve to break down an existing immunity it is very probable that the enormous number of parasites in the infected animal will exert a corresponding action and hence completely overwhelm any resistance which might be produced by the vaccine. Possibly repeated inoculation of large quantities of vaccine might eventually give rise to an amount of antibodies sufficient to check the parasites present.

Before, however, undertaking to put an end to a well developed infection, it was desirable to ascertain whether it was possible to check an infection at the very beginning; in other words, to prevent it by the simultaneous injection of virus and vaccine. In one experiment of this kind, three young rats received an intraperitoneal injection of 0.0001 c.c. of trypanosomal blood. Two of these were reserved for controls while the third was given, likewise intraperitoneally, within an hour, a suspension of the growth from 50 cultures. A fourth rat also received 50 cultures and served as control. It showed a few abortive trypanosomes on the first two days but otherwise no sign of infection. Trypanosomes did not appear in the blood controls until the third day, and then in very small numbers, about one in every 10 fields of the fresh blood. In the treated rat, on the contrary, they appeared on the first day, were two per field on the second, and four per field on the third day. Some blood drawn from the tail of this rat on the second day and injected into another produced a typical infection, thus demonstrating that the trypanosomes present were not of the abortive type. The appearance of the trypanosomes in the treated rat so much earlier than in

the untreated controls is an interesting fact which was corroborated in the second experiment.

Although the trypanosomes in the untreated controls appeared later than in the treated rat, yet, once present, they multiplied more rapidly and in three days were several times more numerous than in the latter. On the seventh day they approximated, roughly speaking, about 200 per field, and this rich infection was maintained during the following three weeks. In the treated rat the maximum number was about 100 per field, on the eighth to the 11th day; the parasites then decreased and disappeared on the 30th day, at which time both controls still showed a maximum infection. This experiment indicated that the injection of a sufficiently large dose of vaccine, shortly after the infective dose, modifies somewhat the course of the infection. The result, however, was not as sharp and conclusive as might be desired.

Consequently, a second experiment was made. Two rats were given an infective dose of 0.0001 c.c., as before. One of these was given 100 cultures in three injections, during the next three hours. In this treated rat, as in the one mentioned above, trypanosomes were found on the first and second days, at which time none could be found in the control. Stained preparations made at this time showed that the trypanosomes were nearly all of the large "female" type, many of which were undergoing active division as indicated by the presence of double nuclei, blepharoplasts, and even of double flagella. Minute flagellates, such as are formed by the breaking up of the eight-celled rosette, were also noted. Of special interest is the fact that though the trypanosomes appeared earlier than in the control they did not continue to increase as they did in the latter.

In the treated rat, the trypanosomes at first appeared to be stimulated to very rapid multiplication, but this was soon checked and the number was greatly decreased, so that on the sixth and seventh days, on an average, not more than one could be found in every two fields of a fresh blood preparation. After this there was a gradual rise and although the rat was examined for 26 days the number never exceeded about 75 per field. The course of the infection in this treated rat, even more so than that in the first experiment, is indicative of a marked inhibitory influence and points to the possi-



bility of aborting an infection by repeated large doses of the vaccine.

The course of the infection in the rats of this last experiment can best be seen from the following summary. The figures here given express roughly the number of trypanosomes present, per field of the No. 7 objective, of a fresh blood preparation. Thus, 1/10 is to read as one trypanosome in 10 fields; 3 as that many per field, etc.

INOCULATED	EXAMINED											
	Feb. 27	Feb. 28	Feb. 29	Mar. 1	Mar. 2	Mar. 3	Mar. 4	Mar. 5	Mar. 6	Mar. 7	Mar. 8	Mar. 9
Treated rat. . . . .	1/10	3	10	10	5	$\frac{1}{2}$	$\frac{1}{2}$	5	10	10	25	50
Control rat. . . . .	0	0	1/20	15	75	100	150	200	200	200	250	Dead

#### PASSIVE IMMUNITY.

Since the animals treated with one or more injections of living cultures develop a very marked immunity, it was to be expected that their blood would carry antibodies, and consequently no special effort has as yet been made otherwise to demonstrate their presence. Three tests, however, of a preliminary character, were made with the blood of rats which had received several spaced injections. These tests, like the curative experiments given above, were intended to ascertain the effect of simultaneous injections of virus and hyperimmune blood. The results, it will be seen, are by no means satisfactory.

*Experiment 1.*—The immunized rat received, in the course of 28 days, seven injections, each of four cultures. After an interval of 153 days, 1 c.c. of blood was drawn from the heart and injected into a clean young rat. This was followed at once by an injection of 0.0001 c.c. of trypanosomal blood. A like dose of the virus was given at the same time to two controls and to the hyperimmune rat. The controls developed the typical rich infection, whereas the latter, as expected, resisted perfectly, although five months had passed since the last protective inoculation. The rat which received the dose of immune blood became infected the same as the controls. Apparently, the blood was without protective action.

*Experiment 2.*—The immunized rat received four injections, of six cultures each. Seventeen days after the last inoculation it was bled and two rats were injected with the blood, receiving 2.0 and 0.5 c.c. respectively. Within a minute, these two rats and two controls were each given 0.0001 c.c. of trypanosomal blood. The rat which received 2 c.c. of the immune blood had a period of incubation of five days; the other three rats showed parasites on the third day. Otherwise, there was no difference, the severity of the infection in the treated rats being the

same as in the controls. Here again, the blood seemed to be without any protective action.

*Experiment 3.*—The immune rat in this test had been given a total of 34 cultures in six injections. It was bled after an interval of 31 days and two rats were given respectively, 3.0 and 1.0 c.c. of its blood. This injection was followed within four minutes by the usual dose of trypanosomal blood (0.0001 c.c.). The same dose of virus was given to two controls and to two young rats born of a mother which had received an injection of two cultures. The latter and the controls gave rich infections. The treated rats, while they possibly showed fewer trypanosomes on the first two days of the infection, eventually gave as rich infections as the controls.

It is evident that in these three tests passive immunity was not conferred. Had the immune blood been injected one or two days previous to the infective dose, the result might have been different. To protect against the virus, when given simultaneously, a much larger dose of blood is needed, or else the blood must come from animals which are more highly immunized.

#### EFFECT OF BERKEFELD FILTRATES OF PLASMOLYZED CULTURES.

Since living as well as dead plasmolyzed cultures immunize against infection with *Tr. lewisi*, it may be pertinent to inquire whether this immunity is due entirely to the solid intracellular constituents or whether soluble products are in any way concerned. In order to obtain some light on this point, a large number of cultures (120) were prepared. The growth was taken up in 0.85 per cent salt solution and transferred to collodium sacs, immersed in running distilled water, and subjected to plasmolysis for 18 hours. At the end of that time the flagellates had disintegrated, or rather shrunk to small rounded forms. The contents of the sacs were then centrifuged, at 8,000 revolutions, to remove the cell detritus, and the clear liquid was passed through a small Berkefeld filter. The filtration, aided by an aspirator, was rapid and required but a few minutes.

The clear filtrate was injected into 6 pairs of rats; the rats of each pair receiving the equivalent of 2, 4, 6, 12, 12, and 24 cultures, respectively. Twelve days later, each of these, as well as four controls, received 0.0001 c.c. of trypanosomal blood. One of the treated rats which received the equivalent of four cultures gave a mild infection which cleared up on the ninth day. All of the other treated rats developed as rich infections as did the controls and,

consequently, the single exception referred to is of no significance. The only conclusion, therefore, which can be drawn from this test is that the Berkefeld filtrates of plasmolyzed cultures have no immunizing properties. It may be added, parenthetically, that the same fact has been noted by Dr. Cumming in connection with his studies (unpublished) on rabic virus. While plasmolyzed suspensions of this virus confer a marked immunity, the filtrates of such suspensions are inert. In other words, it would seem that the insoluble constituents of the cultures, as well as of the rabic virus, are essential to the production of the immunity.

#### SUMMARY.

*Tr. lewisi* after cultivation for several years on rabbit blood agar medium becomes non-infective.

Trypanosomes may appear in very small numbers in the blood of rats inoculated with such cultures but they are incapable of multiplication *in vitro* or *in vivo*.

Rats which receive one or more injections of the living culture acquire a solid immunity which becomes apparent in about 10 days.

The immunity produced by the living culture is lasting and probably persists through life.

The immunity induced by the living vaccine is probably efficacious against the natural transmission through the flea and louse.

The immunity is not due to soluble products, since plasmolyzed and filtered cultures are inert.

This successful immunization against *Tr. lewisi* by means of a living vaccine renders it probable that like results may be obtained with the more strictly pathogenic trypanosomes. It may be added that encouraging results have already been obtained with *Tr. brucei*.

## THE APPLICATION OF THE COMPLEMENT-FIXATION REACTION TO THE DIPHTHERIA GROUP OF ORGANISMS.\*†

By MARY ELIZABETH MORSE.

(From the Laboratory of Pathology [Phillips Fund], Harvard Medical School,  
Boston, Mass.)

The present work forms the second part of a study of the diphtheria group of organisms. The writer is greatly indebted to Dr. H. T. Karsner for advice and the use of the facilities of the experimental division of the laboratory.

The first part of the investigation consisted of a study of the group by the biometrical method.<sup>1</sup> It was found that the group could be divided into two main subgroups, the first comprising the diphtheria bacillus, the second the diphtheroids and Hofmann's bacillus. The second subgroup could be separated further into four species, which are characterized as follows:

*B. hoagii*, a short, thick bacillus, solid, barred, or wedge-shaped, with indistinct granules; growing very heavily on serum with a salmon-pink color, and fermenting dextrose and saccharose, but not maltose or glycerin.

*B. flavidus*, a thick, barred bacillus with large granules; having a heavy yellow or yellow-white growth on serum, and acting on dextrose, glycerin, and maltose, but not on saccharose.

*B. xerosis*, resembling *B. flavidus* morphologically, but giving a meager white growth on serum. It acidifies dextrose, and usually both maltose and saccharose.

*B. hofmanni*, a small, thick, straight bacillus, barred or wedge-shaped, having no granules, and not acting upon dextrose, maltose, glycerin, or saccharose.

\* Received for publication August 3, 1912.

† Aided by a grant from The Rockefeller Institute for Medical Research.

<sup>1</sup> *Jour. Infect. Dis.*, 1912, 11, p. 253.

Complement-fixation in the diphtheria group has been little studied. Lambotte<sup>1</sup> produced strong specific sera in guinea-pigs for the diphtheria bacillus, a "pseudo-diphtheria" bacillus from the normal human conjunctiva, and also one from a membrane on a fowl's eye. He gives no description of these bacilli in the above-mentioned article. He found that both the anti-diphtheroid sera gave partial fixation with the diphtheria bacillus.

Weill-Halle and Bloch-Michel<sup>2</sup> used as antigen a suspension of mucus from the throat in salt solution, and anti-diphtheritic serum as antibody. Their results were positive in 25 cases of diphtheria (controlled by culture), and negative in 10 cases of non-diphtheric angina, nine cases of measles, and three of scarlatina. The authors recommend the complement-fixation reaction as quicker and surer than the microscopic diagnosis, for the early detection of diphtheria.

Priestley<sup>3</sup> attempted to use the complement-fixation reaction for the differentiation of the diphtheria bacillus from the diphtheroids, but could obtain no results because of the lack of satisfactory antigens, although he used both bacterial extracts and bacterial solutions in weak antiformin.

Kolmer,<sup>4</sup> using bacterial extracts, found reciprocal reactions among different strains of diphtheria bacilli and also between the diphtheria bacillus and the Hofmann organism. .

Some work has also been done on the fixation of complement using diphtheria toxin as antigen. Armand-Delille,<sup>5</sup> employing this with anti-diphtheria horse serum, obtained fixation. He found that there were great individual differences in the fixing power of the sera, and that the horses whose sera deviated strongly had shown signs of anaphylaxis during immunization, while those animals whose sera did not deviate had had no "accidents" during treatment. The author also found that there was no relation between the antitoxic and the fixing properties of the sera.

These results were confirmed by Poujol and Delanoe.<sup>6</sup> Cathoire<sup>7</sup>

<sup>1</sup> *Centralbl. f. Bakt.*, 1901, 30, p. 817.

<sup>2</sup> *Soc. méd. d. Hôp. de Paris*, 1910, 29, p. 707.

<sup>3</sup> *Proc. Royal Soc. of Med.*, 1911, 5, p. 146.

<sup>4</sup> *Jour. Infect. Dis.*, 1912, 11, p. 44.

<sup>5</sup> *Mém. de la Soc. de Biol.*, 1908, 65, p. 417.

<sup>6</sup> *Ibid.*, 1908, 67, p. 614.

<sup>7</sup> *Compt. rend. Soc. de Biol.*, 1911, 71, p. 315.

also found that the serum of healthy diphtheria carriers deviated complement in the presence of diphtheria toxin.

In the present experiments the following representative cultures were used for antigens. For the diphtheria bacillus, Park No. 8, the standard antitoxin strain; for *B. hoagii* a strain obtained from Dr. Louis Hoag, and isolated by him from sputum;<sup>1</sup> for *B. flavidus* a culture obtained by Dr. Hoag from the eye; for *B. xerosis* an organism isolated at the Hoagland Laboratory from a septic uterus; and for *B. hofmanni* a culture, "Cameron," obtained by Dr. Anna Williams of the Research Laboratories of the New York Board of Health. A culture "W," also sent by Dr. Williams, was used in addition. This strain, while showing involution forms similar to the Klebs-Loeffler bacillus, also presented characteristics of the Hoag bacillus, i.e., a slightly pink growth on serum, fermentation of saccharose, and absence of action on maltose and glycerin.

In some preliminary work formalized cultures of the bacilli were used as antigens. They were found to be unsatisfactory, however, on account of their marked anti-complementary properties, and bacillary extracts were therefore substituted for them. The method of preparation of these extracts was the same as that employed by Swift and Thoro,<sup>2</sup> with minor changes. The organisms were grown on serum slants in large bottles for four days, this length of time being necessary to secure a sufficient growth of *B. diphtheriae* and *B. xerosis*. The bacillary suspension was washed once. The organisms, after being dried over sulphuric acid, and weighed, were suspended in 10 c.c. of salt solution, containing 0.5 per cent carbolic acid; ground for one hour in an agate mortar with powdered quartz, and shaken for 18 hours in an International Instrument Company centrifuge shaker at about 500 oscillations per minute. A solution containing one milligram of bacterial substance to one cubic centimeter of salt solution was prepared as needed from the concentrated extract.

Specific sera were obtained by the intravenous inoculation of rabbits. Three animals were immunized for each strain, and the

<sup>1</sup> *Boston Med. and Surg. Jour.*, 1907, 157, p. 10.

<sup>2</sup> *Arch. of Int. Med.*, 1911, 7, p. 24.

strongest serum was used for the experiments. Formalized suspensions of the organisms were used, from six to 10 doses being given at intervals of five to seven days. A number of animals succumbed to inoculations with the diphtheria bacillus, so that the treatment had to be conducted very cautiously, beginning with doses of 0.1 c.c. of the suspension. Potent antisera for *B. diphtheriae*, *B. hoagii*, and *B. flavidus* were produced in this way, but no results were obtained with *B. xerosis* and *B. hofmanni*. Immunization with extracts of these two strains was then undertaken, but proved to be equally unsuccessful. The present study is limited, therefore, to the reactions of the three sera, *B. hoagii*, *B. flavidus*, and *B. diphtheriae*, with six antigens.

A rabbit anti-sheep hemolytic system and guinea-pig complement were used.

The method of procedure was in outline as follows:

1. Determination of the strength of the hemolytic serum. The complement was then titrated each day to the serum.
2. Determination of the anti-complementary properties of the antigens.
3. Determination of the fixing dose of each antigen with normal and immune serum.
4. Trial of each immune serum with descending amounts of each antigen, to discover cross-reactions.

The results of the reactions are given in Tables 1, 2, and 3.

From Table 1 it is seen that the serum *B. diphtheriae* gave partial fixation with the homologous antigen in an amount of 0.025 mg. but that it did not react with any of the diphtheroid antigens or with the atypical diphtheria strain "W."

The serum *B. flavidus* (Table 2) fixed completely with its antigen in amounts of 0.025 mg. It also reacted with *B. hoagii*, *B. xerosis*, and the strain "W" at 0.1 mg., and fixed partially with *B. hofmanni* at 0.4 mg. It did not react, however, with the antigen *B. diphtheriae*.

The serum *B. hoagii* fixed partially with its specific antigen in a dose of 0.025 mg. It also fixed with antigen *B. flavidus* and "W" in amounts of 0.05 mg., but did not react with *B. xerosis*, *B. hofmanni*, or *B. diphtheriae*.

TABLE I.  
Fixing Powers of Antigens with Serum *B. diphtheriae*.

ANTIGEN	IMMUNE SERUM <i>B. diph-</i> <i>theriae</i>	NORMAL SERUM	COMPLE- MENT	HEMOLYTIC SERUM	CORPUSCLES 5 PER CENT SUSPENSION	RESULTS	
						Serum <i>B. diphtheriae</i>	Normal Serum
<i>B. diphtheriae</i> — 0.05 mg. .... 0.025 mg. ....	0.1 c.c. "	0.1 c.c. "	2 units "	2 units "	1 c.c. "	Fixation Partial fixation	Hemolysis "
<i>B. hoagii</i> — 0.1 mg. .... 0.05 mg. ....	" "	" "	" "	" "	" "	Hemolysis "	Slight fixation "
<i>B. flavus</i> — 0.4 mg. .... 0.2 mg. .... 0.1 mg. .... 0.05 mg. ....	" " " "	" " " "	" " " "	" " " "	" " " "	Fixation Slight fixation Very slight fixation Hemolysis	Fixation Slight fixation Very slight fixation Hemolysis
<i>B. xerosis</i> — 0.4 mg. .... 0.2 mg. .... 0.1 mg. ....	" " "	" " "	" " "	" " "	" " "	" " "	" " "
<i>B. hofmanni</i> — 0.6 mg. .... 0.4 mg. ....	" "	" "	" "	" "	" "	" "	" "
"W"— 0.2 mg. .... 0.1 mg. ....	" "	" "	" "	" "	" "	Fixation Hemolysis	Fixation Hemolysis
Controls— NaCl 1 c.c. .... NaCl 1 c.c. ....	" "	" "	" "	" "	" "	" No hemolysis	" No hemolysis

Incubated one-half hour at 37.5° C.

Incubated one-half hour at 37.5° C.



TABLE 2.  
Fixing Powers of Antigens with Serum *B. flavidas*.

ANTIGEN	IMMUNE SERUM <i>B. flavidas</i>	NORMAL SERUM	COMPLE- MENT	HEMOLYTIC SERUM	CORPUSCLES 5 PER CENT SUSPENSION	RESULTS	
						Serum <i>B. flavidas</i>	Normal Serum
<i>B. flavidas</i> —							
0.4 mg.....	0.1 c.c.	0.1 c.c.	2 units	2 units	1 c.c.	Fixation	Fixation
0.2 mg.....	"	"	"	"	"	"	Slight fixation
0.1 mg.....	"	"	"	"	"	"	Very slight fixation
0.05 mg.....	"	"	"	"	"	"	Hemolysis
0.025 mg.....	"	"	"	"	"	"	"
<i>B. diphtkeriae</i> —							
0.4 mg.....	"	"	"	"	"	Very slight fixation	Very slight fixation
0.2 mg.....	"	"	"	"	"	Hemolysis	"
<i>B. hoegii</i> —							
0.1 mg.....	"	"	"	"	"	Fixation	Slight fixation
0.05 mg.....	"	"	"	"	"	Hemolysis	Hemolysis
<i>B. xerosis</i> —							
0.4 mg.....	"	"	"	"	"	Fixation	"
0.2 mg.....	"	"	"	"	"	"	"
0.1 mg.....	"	"	"	"	"	"	"
<i>B. hoefmanni</i> —							
0.6 mg.....	"	"	"	"	"	Partial fixation	"
0.4 mg.....	"	"	"	"	"	"	"
0.2 mg.....	"	"	"	"	"	Hemolysis	"
"W"—							
0.2 mg.....	"	"	"	"	"	Fixation	Partial fixation
0.1 mg.....	"	"	"	"	"	"	Slight fixation
0.05 mg.....	"	"	"	"	"	Hemolysis	Hemolysis
Controls— As in Table 1							

Incubated one-half hour at 37.5° C.

Incubated one-half hour at 37.5° C.

TABLE 3.  
Fixing Powers of Antigens with Serum *B. hoagii*.

ANTIGEN	IMMUNE SERUM <i>B. hoagii</i>	NORMAL SERUM	COMPLE- MENT	HEMOLYTIC SERUM	CORPUSCLES PER CENT SUSPENSION	RESULTS	
						Serum <i>B. hoagii</i>	Normal Serum
<i>B. hoagii</i> —							
0.1 mg.....	0.1 c.c.	0.1 c.c.	2 units	2 units	1 c.c.	Fixation	Partial fixation
0.05 mg.....	"	"	"	"	"	"	Slight fixation
0.025 mg.....	"	"	"	"	"	"	Hemolysis
<i>B. diptheriae</i> —							
0.2 mg.....	"	"	"	"	"	Hemolysis	Very slight fixation
0.1 mg.....	"	"	"	"	"	"	Hemolysis
<i>B. flavidus</i> —							
0.4 mg.....	"	"	"	"	"	Fixation	Fixation
0.2 mg.....	"	"	"	"	"	"	Slight fixation
0.1 mg.....	"	"	"	"	"	Partial fixation	Hemolysis
0.05 mg.....	"	"	"	"	"	"	"
0.025 mg.....	"	"	"	"	"	Hemolysis	"
<i>B. zerosis</i> —							
0.4 mg.....	"	"	"	"	"	"	"
0.2 mg.....	"	"	"	"	"	"	"
<i>B. hofmanni</i> —							
0.6 mg.....	"	"	"	"	"	"	"
0.4 mg.....	"	"	"	"	"	"	"
"W"—							
0.2 mg.....	"	"	"	"	"	Fixation	Partial fixation
0.1 mg.....	"	"	"	"	"	"	Slight fixation
0.05 mg.....	"	"	"	"	"	Partial fixation	Hemolysis
Controls— As in Table 1							

## CONCLUSIONS.

The conclusions to be drawn from these experiments are :

1. There are no reciprocal complement-fixation reactions between the typical diphtheria bacillus and representatives of the four species of the diphtheroid group.

2. There are cross-reactions, however, within the diphtheroid group. The species *B. hoagii*, *B. flavidus*, and *B. xerosis* are closely related serologically, as well as morphologically and culturally.

3. *B. hofmanni*, although differing entirely in fermentation powers from the diphtheroids, yet is proved by the complement-fixation test to be related to *B. flavidus*, and therefore must be placed definitely in the diphtheroid group.

4. The action of the culture "W," which is certainly closely allied, both morphologically and culturally, to the diphtheria bacillus, and yet reacts with both *B. hoagii* and *B. flavidus* sera, is further evidence that there are connections between the groups of the diphtheria bacillus and of the diphtheroids.

## THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

### III. THE CHANGES IN THE BLOOD FOLLOWING SPLENECTOMY AND THEIR RELATION TO THE PRODUCTION OF HEMOLYTIC JAUNDICE.\*

BY RICHARD M. PEARCE, M.D., J. H. AUSTIN, M.D., AND  
JOHN H. MUSSER, JR., M.D.

(From the John Herr Musser Department of Research Medicine of the  
University of Pennsylvania, Philadelphia.)

In the first paper<sup>1</sup> of this series certain phenomena have been described which follow splenectomy or are associated with the administration of a hemolytic serum to splenectomized animals. The most striking of these are (1) the frequent failure of jaundice to occur upon the administration of a hemolytic serum during the early period after splenectomy; (2) the increased resistance of the red blood corpuscles, which is evident about one month and more after splenectomy; and (3) the occasional occurrence of spontaneous jaundice in dogs splenectomized for several months. One possible explanation of the first of these phenomena—an influence of the spleen upon the alteration of hemoglobin or its transformation into bile pigment—has been shown, in the second paper<sup>2</sup> of the series, to be untenable. This investigation, moreover, brought out very definite and valuable information concerning the elimination of hemoglobin by the kidney, and the exact relation between hemoglobinemia and jaundice.

In the course of these studies, observations were made which

\* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, July 13, 1912.

<sup>1</sup> Pearce, R. M., Austin, J. H., and Krumbhaar, E. B., I. Reactions to Hemolytic Serum at Various Intervals after Splenectomy, *Jour. Exper. Med.*, 1912, xvi, 363.

<sup>2</sup> Pearce, R. M., Austin, J. H., and Eisenbrey, A. B., II. The Relation of Hemoglobinemia to Hemoglobinuria and Jaundice in Normal and Splenectomized Animals, *Jour. Exper. Med.*, 1912, xvi, 375.

pointed strongly to changes in the blood itself as important factors in the explanation of the phenomena under investigation.

It was noticed, more or less accidentally, that an injection of hemolytic serum into anemic dogs, whether splenectomized or not, did not cause jaundice as readily as in dogs with normal red cell count and normal hemoglobin content.

This observation suggested that it might not be the mere absence of the spleen, but secondary changes in the blood consequent upon the absence of the spleen, that prevented the appearance of jaundice in splenectomized animals. We therefore turned our attention to the condition of the blood in all splenectomized animals. This phase of our problem has already been studied by one of us,<sup>8</sup> who found, in accord with other investigators, that in the dog splenectomy is followed by a gradual progressive decrease in red blood cells and hemoglobin reaching the lowest level about the twenty-sixth day, the hemoglobin varying from 50 to 65 per cent. and the red cells from 2,800,000 to 3,500,000. From this time to the eighty-second day a constant increase in hemoglobin and red cells occurs, the animal not reaching or approximating its normal level, however, until the end of about four and a half months.

A comparison of these phases of blood destruction and regeneration with the various phenomena we had observed, was instructive. The period during which jaundice failed to follow hemolysis, or was slight and evanescent, corresponded roughly to the period, one month, of progressive blood degeneration; the period of increased resistance of the red cells corresponded to the period during which regeneration of blood was occurring, and spontaneous jaundice corresponded, apparently, to the period of more or less complete return of red cells and hemoglobin content to normal.

This correlation suggested the possibility that the failure of jaundice in the early period after splenectomy might be associated in some way with the low hemoglobin content of the red blood cells. Obviously it was possible to test this hypothesis by studying the effect of an hemolytic serum on dogs rendered anemic in some other way than by splenectomy. This was done by bleeding, as shown in

<sup>8</sup> Musser, J. H., Jr., An Experimental Study of the Changes in the Blood Following Splenectomy, *Arch. Int. Med.*, 1912, ix, 592.

the following experiment, in which a normal dog with high hemoglobin content is contrasted with a dog rendered moderately anemic by bleeding.

EXPERIMENT I.<sup>4</sup>

*Effect of Hemolytic Serum on a Dog Rendered Anemic by Bleeding.  
Normal Control.*

Date.	Anemic dog.	Date.	Control dog.
April 18, 1912.	Weight, 10,740 gm. Urine: free of albumin. 179 c.c. of blood taken from jugular vein.	April 16, 1912.	Weight, 5,350 gm. Urine: no albumin, no bile. Blood: red cells, 5,390,000; hemoglobin 107 per cent. Fragility: 0.4 +; 0.5 —.
April 20, 1912.	150 c.c. of blood taken from jugular vein.		Received in vein 0.25 c.c. per kilo of same serum as anemic dog.
April 21, 1912, 10 A. M.	Urine: no albumin, no bile pigment. Blood: red cells, 4,450,000; hemoglobin 87 per cent. Fragility: 0.3 +; 0.4 —.	11.45 A. M.	Much bile in urine.
April 21, 1912, 11.10 A. M.	Received 0.25 c.c. hemolytic serum per kilo, in vein.	2.45 P. M.	Blood: red cells, 5,330,000; hemoglobin 98 per cent.
5 P. M.	Urine: no albumin, no bile. Blood: red cells, 3,400,000; hemoglobin 54 per cent.	3.15 P. M.	Urine: contains hemoglobin and much bile. Blood: red cells, 4,060,000; hemoglobin 85 per cent.
April 22, 1912.	Urine: no hemoglobin, no bile. Blood: red cells, 3,250,000; hemoglobin 49 per cent.	April 17, 1912.	Urine contains hemoglobin and bile pigment. Blood: red cells, 4,470,000; hemoglobin 73 per cent.
April 23, 24, 25, 1912.	Urine: no bile pigment.	April 18, 1912.	Urine: no hemoglobin, but trace of bile.
April 24, 1912.	Blood: red cells, 3,040,000; hemoglobin 42 per cent.	April 19, 1912.	Urine: no bile, no albumin. Blood: red cells, 4,930,000.
April 25, 1912.	Blood: red cells, 2,910,000; hemoglobin 42 per cent.		

In this experiment the anemic dog although it received the same amount of serum proportionately, and on account of its greater weight, twice as much serum actually as the control, failed to develop hemoglobinuria or jaundice. It is true that in as far as the salt solution test can be taken as an indicator, the corpuscles of this

<sup>4</sup>In the experiments described in this paper, all operative procedures were carried out under complete ether anesthesia.

<sup>5</sup>These figures refer to hemolysis in different strengths of salt solution; thus 0.3+ indicates complete hemolysis in 0.3 per cent. salt solution; 0.4 — indicates no hemolysis in 0.4 per cent. solution, and that partial hemolysis occurred in 0.35 per cent. solution.

dog were apparently more resistant than those of the control dog. Nevertheless, the lower hemoglobin content of the blood would appear to be an important factor in determining the occurrence of jaundice. It was at once evident that if it is the condition of the blood and not the absence of the spleen that is important, a recently splenectomized dog with the normal number of red cells and normal hemoglobin content should develop jaundice after the administration of an hemolytic serum. Such a dog was at hand at the time the above experiments were made and was injected with the same serum used in experiment I. As a control, a dog was used that had been splenectomized sixty-six days previously and had recovered from the anemia following splenectomy.

## EXPERIMENT II.

*Effect of Hemolytic Serum on Splenectomized Dogs with Normal Red Cell Count.*

Date.	Four day splenectomy.	Date.	Sixty-six day splenectomy.
April 17, 1912.	Weight, 7,850 gm. Splenectomy.	Feb. 10, 1912.	Splenectomy.
April 21, 1912.	Urine: no albumin, no bile. Blood: red cells, 5,880,000; hemoglobin 102 per cent. Fragility: 0.3 +; 0.45 -.	April 16, 1912.	Weight, 12,680 gm. Urine: faint trace of albumin, no bile. Blood: red cells, 5,230,000; hemoglobin 83 per cent. Fragility: 0.25 +; 0.35 -.
11.25 A. M.	Received in vein 0.25 c.c. serum per kilo (same serum as in experiment I).	12 M.	Received in vein 0.25 c.c. serum per kilo (same serum as in experiment I).
5.20 P. M.	Urine: (by catheter) contains large amount of bile pigment and faint trace of albumin. Blood: red cells, 5,330,000; hemoglobin 93 per cent.	2.45 P. M.	Urine: trace of bile pigment.
April 22, 1912.	Urine: trace of bile. Blood: red cells, 5,260,000; hemoglobin 81 per cent.	3.50 P. M.	Blood: red cells, 5,310,000; hemoglobin 84 per cent.
April 23, 1912.	Urine: faint trace of bile.	11.00 P. M.	Urine: moderate amount of bile pigment.
April 24, 1912.	Urine: no albumin, no bile. Blood: red cells, 4,800,000; hemoglobin 70 per cent.	April 17, 1912.	Urine: moderate amount of bile pigment. Blood: red cells, 4,830,000; hemoglobin 79 per cent.
April 25, 1912.	Blood: red cells, 4,510,000; hemoglobin 80 per cent.	April 18, 1912.	Urine: no bile pigment. Blood: red cells, 4,500,000.
		April 19, 1912.	Urine: no bile.

If the results in these four animals (experiments I and II) are compared, it is seen that jaundice failed to occur only in the animal

## EXPERIMENT III.

*Anemic Splenectomized Dog Controlled by Splenectomized Dog  
with Normal Blood.*

Date.		Date.	
June 2, 1912.	Splenectomy.	May 7, 1912.	Splenectomy.
June 5, 1912.	Blood: red cells, 4,750,000; hemoglobin 82 per cent. Urine: normal, no bile pigment. 1.75 c.c. hemolytic serum injected into vein.	June 5, 1912.	Blood: red cells, 5,230,000; hemoglobin 90 per cent. Urine: normal, no bile pigment. 1.75 c.c. of same serum injected into vein.
June 6, 1912.	Urine: hemoglobin present, no bile pigment.	June 6, 1912.	Urine: hemoglobin present, bile pigment abundant.
June 7, 1912.	Urine: hemoglobin present, no bile pigment. Died.	June 7, 1912.	Urine: hemoglobin present, bile pigment abundant. Experiment discontinued

rendered anemic by bleeding; both splenectomized animals with normal red cell count had a transient but definite elimination of bile pigment in the urine, while the normal control exhibited both hemoglobinuria and jaundice. These observations are of interest in connection with our studies in the second paper of this series. The normal dog suffered a destruction of red cells and reduction of hemoglobin equal to 22 per cent. of the original hemoglobin content. This destruction, as shown by the appearance of hemoglobin in the blood serum two hours and forty minutes after injection, was severe and rapid. The threshold value of the kidney was soon reached and hemoglobin appeared in the urine, but at the same time the amount going to the liver and transformed into bile pigments exceeded the capacity of that organ and resorption caused the pigment to appear in the urine. In the two splenectomized animals, the destruction of hemoglobin and red cells was less, 6 and 16 per cent. respectively, and apparently too slow to cause a concentration of hemoglobin in the blood sufficient to reach the threshold value of the kidney; hemoglobinuria therefore did not occur, but the amount going to the liver was sufficient to lead to jaundice. The slight destruction in the dog that had been splenectomized sixty-six days before is easily explained by increased resistance of the corpuscles; the loss being only 6 per cent. as compared with the anemic dog in



which the loss was 28 per cent., though both had, at the time of injection, practically the same percentage of hemoglobin. The one thing that stands out prominently in these four experiments is the fact that jaundice failed in the dog rendered anemic by bleeding, but occurred in each of the other three dogs with high red cell count.

That this holds true also for the splenectomized anemic dog is shown by experiment III, in which a dog, moderately anemic, three days after splenectomy is controlled by a non-anemic dog which had been splenectomized twenty-six days before.

Here a relatively slight difference in the red cell count and hemoglobin content appears to have been the chief factor in the non-appearance of jaundice.

Another observation, an account of which follows, adds further evidence to support the theory that an hemolytic serum causes jaundice only when the splenectomy is not followed by anemia.

#### EXPERIMENT IV.

A dog weighing 7,730 gm. was splenectomized on March 11. Three days later the urine was normal, the red cells numbered 6,210,000, the hemoglobin content was 92 per cent., and the fragility test gave complete hemolysis in 0.4 per cent. solution. At this time the animal received intravenously 0.5 c.c. per kilo of a very powerful serum, which caused death in less than forty-eight hours. Hemoglobinemia was evident one hour and twenty minutes after injection and hemoglobinuria after four hours; at this time the hemoglobin had dropped to 80 per cent. and at the end of twenty-four hours to 57 per cent. The severe hemoglobinuria rendered tests for bile pigment difficult of interpretation but at autopsy intense bile staining of the skin, sclera, and the mucous membranes and other lighter tissues of the body was found. The abdominal cavity contained a small amount of blood, and petechial hemorrhages studded the peritoneum. The liver showed diffuse necroses and the gall bladder was distended by a thick, black, mush-like bile which would not flow through an incision made in the gall bladder. The lymph nodes of all parts of the body were hemorrhagic. The blood serum contained free hemoglobin.

Another similar experiment follows.

## EXPERIMENT V.

*Effect of Hemolytic Serum Nine Days after Splenectomy, in the Presence of Normal Red Cell Count.*

Date.	Dog 28, weight 10.015 gm.	Date.	Dog 37, weight 8.255 gm.
March 5, 1912.	Urine normal.		
March 6, 1912.	Splenectomy.		Control.
March 8, 1912.	Blood: red cells, 7,620,000; hemoglobin 102 per cent.		
March 13, 1912.	Blood: red cells, 6,540,000; hemoglobin 105 per cent. Fragility: 0.25 +; 0.35 -.		
March 14, 1912.	Urine: faint trace of albumin, no bile pigment.	March 15, 1912.	Urine: normal; no bile pigment.
March 15, 1912.	Urine: faint trace of albumin, no bile pigment.	3.15 P. M.	Blood: red cells, 5,440,000; hemoglobin 90 per cent. Fragility: 0.35 +; 0.45 -.
1.30 P. M.	Blood: red cells, 6,260,000; hemoglobin 98 per cent.	3.30 P. M.	0.3 c.c. of same serum per kilo in vein.
1.55 P. M.	0.3 c.c. serum per kilo in vein.	4.05 P. M.	Blood serum free from hemoglobin.
3.35 P. M.	Blood serum clear.	5.45 P. M.	Urine by catheter deeply bile stained, and contains much coagulable protein.
5.15 P. M.	Urine: by catheter, deeply bile stained, considerable coagulable protein; no hemoglobin. Red cells: 5,490,000; hemoglobin 100 per cent. Blood serum: clear.		Blood: red cells, 5,720,000; hemoglobin 85 per cent.; serum shows doubtful trace of hemoglobin.
March 16, 1912, 9 A. M.	Urine: flocculent precipitate of albumin, large amount of bile pigment; no hemoglobin.		Urine: highly colored by bile pigment, trace of albumin; no hemoglobin.
2 P. M.	Urine: no hemoglobin, much bile pigment, albumin present.	March 16, 1912, 5.45 P. M.	Red cells, 4,480,000; hemoglobin 67 per cent. Urine: bile pigments lessening. Blood: hemoglobin 52 per cent.
3.30 P. M.	Red cells, 3,600,000; hemoglobin 72 per cent.		Urine: faint trace of bile pigment. Blood: red cells, 2,320,000; hemoglobin 45 per cent.
March 17, 1912.	Urine: no hemoglobin, bile present, albumin present.		Urine: faint trace of bile. Blood: red cells, 3,020,000; hemoglobin 40 per cent.
3 P. M.	Blood: hemoglobin 50 per cent.		Blood: serum contains no free hemoglobin.
March 18, 1912.	Urine: trace of albumin, bile pigments diminishing in amount. Blood: hemoglobin 37 per cent.		
March 19, 1912.	Blood: hemoglobin 40 per cent.; red cells, 3,170,000. Serum shows no free hemoglobin. Urine: high colored, no albumin, good reaction for bile pigments.		
March 20-27, 1912.	Urine: contains bile pigment in small amounts.	March 20 and 21, 1912.	Urine: contains trace of bile pigments and albumin.
March 21, 1912.	Blood: red cells, 2,350,000; hemoglobin 31 per cent.		

EXPERIMENT V (*Continued*).

Date.	Dog 28, weight 10,015 gm.	Date.	Dog 37, weight 8,255 gm.
March 23, 1912.	Blood: red cells, 2,500,000; hemoglobin 35 per cent. Fragility: 0.25 +; .35 —.	March 21, 1912.	Blood: red cells, 3,040,000; hemoglobin 33 per cent.
March 26, 1912.	Blood: d cells, 3,260,000; hemoglobin 51 per cent. Experiment discontinued.	March 23, 1912.	Urine no albumin, no bile pigment. Died.

## SPLENECTOMIZED ANIMALS WITH INCREASED RESISTANCE OF CORPUSCLES.

That the red cells of splenectomized animals after one month and more have a true resistance to hemolytic serum and that this is not merely a matter of test-tube experiment is shown in experiment VI.

## EXPERIMENT VI.

*Increased Resistance Forty Days after Splenectomy.*

Date.		Date.	
Feb. 8, 1912.	Splenectomy.		Control.
March 11, 1912.	Weight, 6,780 gm. Urine: normal. Blood: red cells, 5,560,000; hemoglobin 65 per cent. Fragility: 0.3 +; 0.4 —.		
March 19, 1912.	Urine: normal. Blood: red cells, 5,710,000; hemoglobin 82 per cent.	March 11, 1912.	Urine: normal. Blood: red cells, 6,220,000; hemoglobin 102 per cent. Fragility: 0.4 +; 0.5 —.
4.30 P. M.	0.5 c.c. serum per kilo in vein.		
6 P. M.	Serum faintly tinged with hemoglobin.	2.35 P. M.	0.5 c.c. per kilo of same serum in vein.
9.30 P. M.	No urine in bladder. Blood: red cells, 5,550,000; hemoglobin 65 per cent.	4.02 P. M.	Serum tinged with hemoglobin.
		6.30 P. M.	No urine in bladder. Serum deeply stained with hemoglobin.
March 20, 1912.	Died. Urine from bladder shows much albumin, trace of bile; no hemoglobin.	12 P. M.	Red cells, 4,330,000; hemoglobin 94 per cent. Died during night; no urine.

These animals died as the result of a toxic property distinct from the hemolytic activity of the serum, but the difference in the resistance of the red cells to the hemolytic agent is clearly shown.

Still more striking is the very slight destruction of red cells in an animal that developed spontaneous jaundice seven and a half months after splenectomy, as shown in experiment VII.

## EXPERIMENT VII.

*Increased Resistance Seven and a Half Months after Splenectomy.*

Date.	Dog 32, <sup>a</sup> weight 9,170 gm.	Date.	Dog 33, weight 7,050 gm
July 19, 1911.	Splenectomy.		Control.
March 7, 1912.	Much bile pigment in urine.		Urine: normal.
March 8, 1912.	Much bile pigment in urine.		Urine: normal.
11 A. M.	Blood: red cell count 6,120,000; hemoglobin 97 per cent.	10.45 A. M.	Blood: red cell count, 6,610,000; hemoglobin 108 per cent.
2.45 P. M.	Red cells hemolyzed completely by 0.3 per cent. salt solution.		
2.47 P. M.	0.5 c.c. serum per kilo in vein.	2.16 P. M.	Red cells hemolyzed completely by 0.4 per cent. salt solution.
3.02 P. M.	No free hemoglobin in serum.		
3.18 P. M.	No free hemoglobin in serum.		
3.38 P. M.	No free hemoglobin in serum.		
5.15 P. M.	Urine free of hemoglobin.	2.18 P. M.	0.5 c.c. of same serum per kilo in vein.
5.30 P. M.	Blood count: red cells, 6,480,000; hemoglobin 101 per cent.		
March 9, 1912.	Urine: no hemoglobin, but much bile pigment.	2.32 P. M.	No free hemoglobin in serum.
2.15 P. M.	Blood count 6,800,000; hemoglobin 102 per cent.	2.53 P. M.	Trace of hemoglobin in serum.
2.30 P. M.	Received in vein 8.5 c.c. of same serum (nearly 1 c.c. per kilo).	3.10 P. M.	Trace of hemoglobin in serum.
2.32 P. M.	No hemoglobin in serum.	5.10 P. M.	Urine contains hemoglobin.
2.55 P. M.	No hemoglobin in serum.		
5.00 P. M.	Trace of hemoglobin in serum.	5.10 P. M.	Blood count, 3,580,000; hemoglobin 103 per cent.
5.00 P. M.	Hemoglobin in urine in very small amount but positively determined by spectroscope.		
5.00 P. M.	Red cells, 5,200,000; hemoglobin 95 per cent.	March 9, 1912.	Urine: very marked hemoglobinuria: bile test positive.
March 10, 1912.	Marked hemoglobinuria.		No second injection.
March 11, 1912.	Died.	March 11, 1912.	No hemoglobin in urine, but trace of bile.

Here again the increased resistance of the red cells of the splenectomized animal is very marked. Equal doses of serum failed to cause marked hemolysis in the splenectomized animal, as determined by blood count and absence of hemoglobin in serum and urine. An extraordinarily large dose, however, caused severe blood destruction and death after forty-eight hours. On the other hand, the first dose caused in the control animal marked hemoglobinuria and great destruction of red cells.

<sup>a</sup>One of the dogs (No. 2) used by J. H. Musser, Jr., in his "Experimental Study of the Changes in the Blood Following Splenectomy," *Arch. Int. Med.*, *loc. cit.*

**THE CONDITION OF THE BLOOD IN ANIMALS WITH SPONTANEOUS JAUNDICE.**

The observation that animals with low red cell count and low hemoglobin, irrespective of the presence or absence of the spleen, failed to develop jaundice after the administration of a hemolytic serum, and the obverse, that this serum in splenectomized animals with normal blood count and hemoglobin content always caused jaundice, suggested the possibility that the spontaneous jaundice, occasionally occurring after splenectomy, might be associated in some way with an over-regeneration of blood leading to an abnormally high red cell count and hemoglobin content. With this possibility in view blood examinations have been made in all long period experiments. The data at hand, though meager, are suggestive of a possible relation between the jaundice and the condition of the blood.

Well marked choluria following splenectomy, and occurring without administration of a hemolytic poison, has been seen in two animals, and slight choluria likewise in two. In one of the former the blood was not examined; in the second (experiment VII), in which a very marked choluria was found seven and a half months after operation, the red cell count was 6,120,000, and the hemoglobin 97 per cent. So also in one of the animals with slight choluria, the blood count was 6,220,000, and hemoglobin 103 per cent.; in a second the red count was 5,740,000.

These were animals which had recovered from the temporary anemia due to splenectomy and possessed the normal number, or perhaps a slight excess of red blood cells. On the other hand, in the only animal which was carried for a long period (ten months) with careful observation of the blood and which did not develop choluria, the blood counts were low, varying during the last two months from 4,460,000 to 4,970,000, and with hemoglobin ranging from 68 to 81 per cent.

These observations are too few in number and not sufficiently controlled to allow definite conclusions but they suggest the possibility that spontaneous jaundice after splenectomy may be associated in some way with the period of complete regeneration of the blood.<sup>7</sup>

<sup>7</sup> This suggestion we offer with some hesitancy, for recently we have observed in five apparently normal dogs an unexplainable spontaneous jaundice.

#### SUMMARY.

The results of this study of the changes in the blood after splenectomy and of the effect of the administration of hemolytic serum allow the following tentative conclusions:

1. The failure of a hemolytic serum to cause jaundice is due in some way to the anemia which frequently follows splenectomy. Animals, whether splenectomized or not, which have a low blood count and low hemoglobin content do not readily develop jaundice after administration of a hemolytic serum. On the other hand, in animals with a normal blood picture such a serum readily causes, during early periods after splenectomy, a well marked jaundice.

2. The difficulty of producing hemoglobinemia and jaundice in animals splenectomized one month or more, is due, as shown by blood count, the use of hypotonic salt solution test, and by the search for the presence of hemoglobin in the serum and urine, to an increase in the resistance of the red cells. The results of detailed serologic tests upon this point will be presented later.<sup>8</sup>

3. It is possible that spontaneous jaundice occurring at long periods after splenectomy is an accompaniment of the complete regeneration of the blood. The study of the blood in animals splenectomized for long periods, seven to ten months, indicates that spontaneous jaundice occurs only when the animal has recovered from the initial period of anemia and has a high red cell count and high hemoglobin content.

4. As to the mechanism responsible for the anemia following splenectomy and for the increased resistance of the red cells, we can at present offer no explanation.

<sup>8</sup> Karsner, H. T., and Pearce, R. M., IV. A Study, by the Methods of Immunology, of the Increased Resistance of the Red Blood Corpuscles after Splenectomy, *Jour. Exper. Med.*, 1912, xvi, 769.

## THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

### IV. A STUDY, BY THE METHODS OF IMMUNOLOGY, OF THE INCREASED RESISTANCE OF THE RED BLOOD CORPUSCLES AFTER SPLENECTOMY.\*

BY HOWARD T. KARSNER, M.D., AND RICHARD M. PEARCE, M.D.

(From the John Herr Musser Department of Research Medicine of the  
University of Pennsylvania, Philadelphia.)

In the first and third communications of this series<sup>1</sup> it was shown that a dog that has been splenectomized for some time is more resistant to a specific hemolytic immune serum than is the non-splenectomized dog, and that to produce hemoglobinemia and hemoglobinuria, or jaundice, in such an animal it is necessary to administer larger amounts of serum than in the normal animal. Tests *in vitro* with hypotonic salt solution and with hemolytic serum showed that this diminished susceptibility of the animals to the action of a hemolytic serum was in all probability due to an increased resistance of the red cells. These tests, however, were made irregularly and were but incidental to other phases of the general investigation. In the present communication the results of a special study of this problem by the accurate and detailed methods of immunology are presented in detail.

Increase in resistance of the red cells to hemolytic agents after splenectomy is not a new observation. It has been noted in the dog by Bottazzi,<sup>2</sup> Banti,<sup>3</sup>

\* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, July 13, 1912.

<sup>1</sup> Pearce, R. M., Austin, J. H., and Krumbhaar, E. B., I. Reactions to Hemolytic Serum at Various Intervals after Splenectomy, *Jour. Exper. Med.*, 1912, xvi, 363; Pearce, R. M., Austin, J. H., and Eisenbrey, A. B., II. The Relation of Hemoglobinemia to Hemoglobinuria and Jaundice in Normal and Splenectomized Animals, *ibid.*, 375; Pearce, R. M., Austin, J. H., and Musser, J. H., Jr., III. The Changes in the Blood Following Splenectomy and Their Relation to the Production of Hemolytic Jaundice, *ibid.*, 758.

<sup>2</sup> Bottazzi, F., La milza come organo emocatatonistico, *Sperimentale, Sez. biol.*, 1894, xlviii, 433.

<sup>3</sup> Banti, G., La milza nelle itterizie pleiochromiche, *Gaz. d. osp.*, 1895, xvi, 489.

Vast,<sup>4</sup> Pugliese and Luzzatti,<sup>5</sup> and Joannovics,<sup>6</sup> in the guinea pig by Gabbi,<sup>7</sup> and in the rabbit by Domenicci.<sup>8</sup> The hemolytic agent used by these investigators was, as a rule, toluylendiamine, though occasionally pyrodin (Pugliese and Luzzatti), acetylphenylhydrazine (Banti), and specific hemolytic immune serum (Joannovics) have been employed. In general it has been assumed that the increased resistance to the toxic influence of the drug, or the failure to produce hemoglobinemia or jaundice, is due to increased resistance of the red cells. Banti invokes also the absence of the alleged hemolytic function of the spleen, and Vast also believes that for the destruction of red cells the intervention of the spleen is necessary. This point of view is to some extent supported by Joannovics. Pugliese and Luzzatti, on the other hand, deny the influence of the absence of the spleen and point to the presence, after repeated doses of pyrodin, of newly formed immature nucleated corpuscles, poor in hemoglobin, which they think may possibly be more resistant to pyrodin than are the normal cells. Few exact studies of the condition of the corpuscles have been made, and these are limited, as in the investigations of Brissaud and Bauer,<sup>9</sup> Chaler and Charlet,<sup>10</sup> and Pel,<sup>11</sup> to the use of hypotonic salt solutions. No careful study of the resistance of these cells to hemolytic serum *in vitro* has been made, and the allied problems of possible antihemolytic action of the serum and of possible changes in complement content have not been investigated.

Brissaud and Bauer, working with two rabbits and using hypotonic salt solution of varying strengths, found a decreased resistance of the red cells during eight to ten days after splenectomy with, "after the lapse of time," a return to normal, but no increase in resistance. They do not, however, state how long the experiment was continued. Chaler and Charlet, using both the rabbit and the dog, and the salt solution test, conclude that splenectomy is followed apparently by a slight increase in the resistance of the red cells. Pel, in a very extensive study, found that as an average of fifty-eight determinations on normal dogs the first trace of hemolysis occurred in 0.42 per cent. salt solution as compared with 0.35 as the average for thirty observations on splenectomized dogs; the average concentration at which hemolysis was complete was, for normal dogs, 0.30 per cent. salt solution, and for splenectomized dogs 0.23 per

<sup>4</sup> Vast, cited by Joannovics, *loc. cit.*; original not available.

<sup>5</sup> Pugliese, A., and Luzzatti, T., Contribution à la physiologie de la rate, *Arch. ital. de biol.*, 1900, xxxiii, 349.

<sup>6</sup> Joannovics, G., Experimentelle Untersuchungen über Ikterus, *Ztschr. f. Heilk., Abt. f. path. Anat.*, 1904, xxv, 25.

<sup>7</sup> Gabbi, U., Ueber die normale Hämatolyse mit besonderer Berücksichtigung der Hämatolyse in der Milz, *Beitr. z. path. Anat. u. z. allg. Path.*, 1893, xiv, 351.

<sup>8</sup> Domenicci, cited by Pel, *loc. cit.*; original not available.

<sup>9</sup> Brissaud and Bauer, Recherches sur la résistance des globules rouges chez le lapin, *Compt. rend. Soc. d. biol.*, 1907, lxii, 1068.

<sup>10</sup> Chaler, J., and Charlet, L., État de la résistance globulaire chez l'animal normal et splénectomisé, *Jour. de physiol. et de path. gén.*, 1911, xiii, 728.

<sup>11</sup> Pel, L., Über die Resistenz der roten Blutkörperchen gegenüber hypotonischen Kochsalzlösungen bei entmilzten Hunden, *Deutsch. Arch. f. klin. Med.*, 1912, cvi, 592.



cent. Thus in both series of observations the increased resistance of the splenectomized animals was the equivalent of 0.07 per cent. salt solution. The difference may be expressed in another way; in the fifty-eight observations on normal animals hemolysis began in all but one test in solutions of 0.48 to 0.40 per cent., while of thirty observations on splenectomized animals, in all but two it began in solutions of 0.38 to 0.30 per cent.; likewise complete hemolysis occurred in the normal group only seven times below 0.30, while in the splenectomized group it occurred always below 0.30 per cent. solution. The resistance of the red cells was found to increase gradually and reach its maximum after about two months; further lapse of time showed no tendency to return to normal. The longest period after splenectomy was two years and four months.

Pel makes a general statement concerning the influence of the serum to the effect that the serum of a splenectomized dog added to the red cells of a normal dog does not increase the resistance of the latter to hypotonic salt solution, and *vice versa*, that the addition of normal serum to the red cells of a splenectomized dog does not decrease their resistance.

Blood counts showed a slight decrease in the number of red cells after splenectomy but not enough in the opinion of Pel, in view also of only slight changes in the percentage of hemoglobin, to have any relation to the increased resistance of the red cells. As to the factors responsible for the increased resistance, Pel offers no explanation.

#### EXPERIMENTAL PART.

In the present investigation we have used six dogs. One had been splenectomized ten days, a second, thirty days, and a third, four months before. The last animal had been given hemolytic immune serum two months before the beginning of the immunological tests, and therefore as a control for this animal, a dog which had received hemolytic immune serum five weeks previously, but was otherwise normal, was included in the series, and as controls for the other two animals two normal dogs were used.

For the determination of the resistance of the corpuscles to hypotonic salt solution we employed, with slight modifications, the method of Theobald Smith<sup>12</sup> as used by Gay.<sup>13</sup> Chemically pure sodium chloride was dried for two hours at 170° C. and immediately weighed in amounts necessary to make 500 cubic centimeter volumes of salt solution ranging from 0.1 to 0.5 per cent. in steps of 0.025 per cent. In order to be sure of approximately the same

<sup>12</sup> Smith, T., and Brown, H. R., The Resistance of the Red Corpuscles of the Horse to Salt Solutions of Different Tonicities, *Jour. Med. Research*, 1906, xv, 425.

<sup>13</sup> Gay, F. P., The Function of Tonicity in Human Isohemagglutination, *Jour. Med. Research*, 1907-08, xvii, 321.

TABLE I.

	Percentage strength of salt solutions. (June 10, 1912)	0.275	0.300	0.325	0.350	0.375	0.400	0.425	0.450	0.475	0.500	0.525
Corpuscles of	Dog 30. Normal.....	100	100	100	100	100	100	100	60	40	Trace	0
	Dog 53. Normal.....	100	100	100	100	100	80	40	20	Trace	0	0
	Dog 51. Splenectomy, May 31.....	100	100	100	80	60	20	Trace	Trace	0	0	0
	Dog 46. Splenectomy, May 10.....	100	100	100	100	80	60	40	20	Trace	0	0
	Dog 24. Splenectomy, Feb. 10.											
	Immune serum, April 16.....	100	100	80	40	20	Trace	Trace	0	0	0	0
Dog. 43	Immune serum, April 21.....	100	100	100	100	80	60	20	Trace	0	0	0

volume of corpuscles in the anemic as in the normal bloods, the gently defibrinated blood was centrifuged and the serum drawn off. 0.1 of a cubic centimeter of the corpuscular mass was measured accurately in a graduated pipette and placed in three cubic centimeters of each of the various salt solutions. Standard colorimetric scales for comparison were made by laking red cells with distilled water; thus the laking of 0.4 of a cubic centimeter of the corpuscular mass in twelve cubic centimeters of distilled water represented a standard of 100 per cent. hemolysis. Dilutions of this solution were made so as to have tubes showing the color values of 80, 60, 40, and 20 per cent. hemolysis. Less than 20 per cent. hemolysis is indicated in table I as a trace of hemolysis. In most instances this scale was entirely satisfactory, but occasionally, although a tube showed 100 per cent. hemolysis colorimetrically, there was, on shaking, a slight macroscopic sediment of incompletely hemolyzed corpuscles; this result is indicated by a minus sign after the approximate percentage of hemolysis. Upon adding the corpuscles to the salt solution, a preliminary reading was made and the mixtures were placed in the refrigerator. The final reading at the end of eighteen hours was made the basis for table I.

It will be seen that the blood of the normal dogs (30 and 53) shows hemolysis in fairly high percentages of salt solution, but that the resistance is increased in all the abnormal animals. It is true that dog 46, a splenectomized animal, shows initial hemolysis in the same percentage of salt solution as normal dog 53; but inspection will show that whereas in the normal dog hemolysis is complete at 0.350 per cent., it is not complete in dog 46 until 0.300 per cent. is reached. There can be no doubt that the cells of dog 24, the animal which had been longest splenectomized, show the greatest degree of resistance. That this resistance is due for the most part, if not entirely, to splenectomy, is, in view of results with bloods 30 and 53, most probable. On the other hand, it is evident, as shown by the experience with blood 43, that the administration of a hemolytic immune serum is followed by an increased resistance of the red cells in the absence of splenectomy.

In order to determine the resistance of the corpuscles to a specific

hemolytic immune serum<sup>14</sup> the following technique was employed. The corpuscles were washed three times in 0.85 per cent. salt solution, and blood suspensions were made of 5 per cent. of red cells as contained in the centrifuged corpuscular mass. The latter rather than whole blood was used because the use of whole blood would be fallacious in the case of anemic animals. The immune serum was titrated against normal corpuscles, guinea pig complement was used in doses of 0.1 of a cubic centimeter, and the experiment arranged as indicated in table II.

TABLE II.

	Dilutions of immune serum. (June 14, 1912)		1/20	1/50	1/100	1/150	1/200	1/250	1/300	Comple- ment control.	Ambo- ceptor control.	Blood control
Washed corpuscle suspensions of	Dog 30.	Normal. ....	C.H. <sup>14</sup>	C.H.	H.	H.	P.H.	P.H.	P.H.	o	o	o
	Dog 53.	Normal. ....	C.H.	C.H.	H.	H.	P.H.	P.H.	P.H.	o	o	o
	Dog 51.	Splenectomy, May 31 ..	P.H.	P.H.	P.H.	P.H.	P.H.	P.H.	P.H.	o	o	o
	Dog 46.	Splenectomy, May 10 ..	P.H.	P.H.	P.H.	P.H.	P.H.	P.H.	P.H.	o	o	o
	Dog 24.	Splenectomy, Feb. 10. Immune serum, April 16 ..	P.H.	P.H.	P.H.	P.H.	P.H.	o	o	o	o	o
	Dog 43.	Immune serum, April 21 ..	P.H.	P.H.	P.H.	P.H.	P.H.	P.H.	P.H.	o	o	o

Technical limitations prevent, in this experiment, as close an estimation of resistance as is possible with hypotonic salt solution, but it can be seen readily that whereas dilutions of 1/20 and 1/50 produced complete hemolysis of normal corpuscles, the corpuscles of the abnormal animals were resistant to these dilutions. The fact that the corpuscles of dog 24, which had been splenectomized four months previously, were most resistant is shown by the fact that whereas partial hemolysis appeared in all other corpuscles in dilutions of 1/250 and 1/300, the corpuscles of this dog resisted completely hemolysis at such dilutions. Here again the results with the blood of dog 43 demonstrate that the administration of an hemolytic

<sup>14</sup> Prepared by injecting the rabbit five times with 5 c.c. of dog blood, at intervals of five to seven days.

<sup>15</sup> In the tables, C.H. = complete hemolysis; H. = hemolysis; P.H. = partial hemolysis.

serum increases the resistance of the red cells, irrespective of splenectomy.

Since it has been shown by Karsner and Pearce<sup>16</sup> that agglutination limits hemolysis, it was considered wise to avoid possible error due to such action. The agglutinative effect of the immune serum was therefore examined in the same dilutions as were used for hemolysis. It was found that the agglutinability of the corpuscles was the same for all bloods except that of dog 43, the corpuscles of which formed somewhat smaller and less firm clumps than the others, but agglutinated at the same time and in the same dilutions as the rest. It was thus conclusively shown that the variations in resistance to hemolysis do not depend on variability of agglutination.

The fact that washed corpuscles were used in the experiments with immune serum would indicate that the increase in resistance is indeed a property of the corpuscles themselves, but in order to prove this point absolutely, the sera of all six dogs were examined for antihemolytic properties. It has been shown by Karsner and Pearce<sup>17</sup> that fresh dog serum has a definite antihemolytic property in an homologous hemolytic system. In the experiments designed to determine whether this property is augmented after splenectomy, an immune serum was carefully titrated with a constant dose of guinea pig complement. Two rows of tubes were used; in the tubes of one row were placed 0.5 of a cubic centimeter of a dilution of dog serum, as indicated in table III, and one dose (0.5 of a cubic centimeter) of amboceptor. In the second row the same amount of dog serum and two doses of amboceptor were placed. The tubes were then incubated for one half hour, and to the first row one dose of complement was added, to the second row two doses of complement, and to all the tubes the proper amount of 5 per cent. blood suspension. The second incubation lasted one hour and the readings were found to be uniform throughout. The results are shown in table III, in which, however, in order to avoid unnecessary duplication, the results with two sera only are given.

<sup>16</sup> Karsner, H. T., and Pearce, R. M., The Antibodies Produced by Various Constituents of Dog's Bile, *Jour. Med. Research*, 1912, xxvi, 357.

<sup>17</sup> Karsner, H. T., and Pearce, R. M., *loc. cit.*

TABLE III.

Serum dilutions. (June 15, 1912)		1/2	1/4	1/8	1/16	1/32	1/64	0
Serum of dog 30 (normal) in dilutions indicated plus	1 dose complement	P.H.	P.H.	P.H.	P.H.	P.H.	P.H.	P.H.
	1 dose amboceptor	P.H.	P.H.	P.H.	P.H.	P.H.	P.H.	P.H.
	2 doses complement	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
Serum of dog 24 (splenectomy Feb. 10, 1912. Immune serum April 16) in dilutions indicated plus	2 doses amboceptor	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
	1 dose complement	P.H.	P.H.	P.H.	P.H.	P.H.	P.H.	C.H.
	1 dose amboceptor	P.H.	P.H.	P.H.	P.H.	P.H.	P.H.	C.H.
	2 doses complement	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
	2 doses amboceptor	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

It is seen that in all the dilutions of dog serum used, the action of one dose of amboceptor and of complement was hindered, but in none of the dilutions was the antihemolytic property sufficient to hinder the action of two doses of complement and of amboceptor. Weaker dilutions were not considered necessary because it seems certain that between the action of a dilution of 1/64 on one dose of hemolysin and of whole serum on two doses, no fine gradation could exist.

In determining the complement value of dog serum, it was thought best to use a sheep hemolytic system. The anti-sheep amboceptor was titrated with normal dog complement and the approximate complement value determined. Then two series of tubes were arranged with one dose of amboceptor, the proper amount of 5 per cent. sheep blood suspension, and in the first row increasing doses of fresh serum from the six dogs. The second row was placed so that each tube contained corresponding ingredients to those in the first row except that no anti-sheep amboceptor was present. The latter series showed the natural amboceptor in the dog serum. It was found that the natural sheep hemolysin was the same in all except one. In spite of this the complement value was precisely the same in all six animals. The results with one animal are charted (table IV) to show the technique.

TABLE IV.

Dog complement in c.c. (June 15, 1912)		0.01	0.02	0.04	0.07	0.1	1.5
Dog (normal) ..	with amboceptor .....	0	P.H.	H.	C.H.	C.H.	C.H.
	without amboceptor .....	0	0	Trace	H.	C.H.	C.H.

## DISCUSSION.

These studies demonstrate conclusively that the increased resistance of the erythrocytes of the splenectomized dog depends upon the corpuscles rather than the serum. Pel's experiments demonstrated this fact in so far as the resistance to hypotonic salt solutions is concerned, and the experiments reported in this communication not only confirm Pel's observations but add positive evidence of resistance to immune serum; and they demonstrate, moreover, that the serum of the splenectomized animal presents no change in complementary activity and no distinct increase in antihemolytic property. This demonstration is conclusive, for in the hemolysis *in vivo* as well as *in vitro* three components are necessary; the corpuscles, the amboceptor, and the complement. If anticomplementary substances were present in the blood, they would be effective in diminishing the complementary action of the animal's serum. If the complement itself were small in amount or diminished in activity, it would be seen in the results of the test-tube experiments. The amboceptor is the constant and controllable factor in the experiment and the only uncontrollable agent in this connection is an anti-amboceptor or antihemolysin, which, from the results of our experiments, is not found in the three splenectomized animals, or in the animal which had been given immune serum, in greater amounts than in normal dogs. The remaining factor is the red blood corpuscle itself; and that it is in this cell that the increased resistance lies is shown by these experiments, which demonstrate beyond question that the corpuscles of the splenectomized animals and of the animals which had been given immune serum exhibit distinctly increased resistance to the definitely controlled action of specific amboceptor and complement.

Why the absence of the spleen increases the resistance of the red cells is still an open question, but the information obtained by this study, excluding as it does changes in the serum, narrows the problem to the red cells themselves. Of great significance are the results of the study of the blood of dog 43. This blood was used as a control on the supposition that an administration of a hemolytic serum might have caused the production of an antihemolysin. But this was not demonstrable either in this dog or in dog 24, which also had received hemolytic serum.

The conclusion is unavoidable that the increased resistance of the corpuscles, after the use of such a serum, is associated with the anemia caused by the hemolytic serum. This increased resistance of red cells in anemia is not uncommon, and, as has been stated, has been brought forth by Bottazzi, in connection with the action of pyrocin, as an explanation of the increased resistance of the red cells to this drug after splenectomy. That the increased resistance may be due to splenectomy alone and be entirely independent of the action of hemolytic substance introduced from without, is shown in the experiments with the blood of dogs 51 and 46, and has also been clearly demonstrated in the first and third communications of this series. One cannot therefore escape from the conclusion that the increased resistance of the red cells is a concomitant of the regeneration of the blood which follows the anemia caused by the removal of the spleen. That anemia does follow splenectomy has been shown in many investigations, and recently has been demonstrated anew in this laboratory by Musser;<sup>18</sup> that the return of the blood picture to normal is accompanied by increased resistance of the red cells to hemolytic sera has been shown in the third communication of this series, in which also it has been demonstrated that anemia has an important bearing on the production of hemolytic jaundice.

The main problem therefore is the explanation of the anemia following splenectomy. The possibility that this anemia may be due to an autohemolysin normally neutralized by the spleen, but operative in the absence of the spleen, is excluded by the control experiments here presented. There remains therefore the question of how the spleen regulates or controls or is otherwise concerned in the general process of blood destruction and regeneration, and to obtain information on this point, the changes in the lymph nodes and bone marrow, after splenectomy, are now receiving especial consideration.

#### CONCLUSIONS.

1. The erythrocytes of splenectomized dogs show increased resistance to the action of hypotonic salt solutions and to specific

<sup>18</sup> Musser, J. H., Jr., An Experimental Study of the Changes in the Blood Following Splenectomy, *Arch. Int. Med.*, 1912, ix, 592.



hemolytic immune serum. The degree of resistance appears to increase with the length of time that has elapsed after splenectomy.

2. This increased resistance of the erythrocytes is not due to an increased antihemolytic power of the animal's serum or to a diminished complementary value of the serum, but is a property depending upon the erythrocytes themselves.

3. Non-splenectomized animals receiving a single injection of specific hemolytic immune serum and developing a temporary anemia show likewise on recovery an increased resistance of the corpuscles without the presence of antihemolysin in demonstrable amount.

4. As anemia of varying grade is a characteristic result of splenectomy, it would appear that the increased resistance of the corpuscles is a concomitant of the regeneration of the red cells following such anemia and is thus analogous to the increased resistance of such cells not infrequently observed in various forms of experimental anemia.

5. There is no evidence to indicate that the anemia after splenectomy is due to the presence of hemolytic bodies, or that the increased resistance of the cells is due to antihemolytic bodies, accumulating in the serum as the result of the ablation of the spleen. It is evident therefore that the spleen in some way controls or regulates blood destruction (and regeneration ?), and in the hope of throwing light on the subject, an investigation of the bone marrow and lymph nodes of splenectomized dogs is now under way.

## THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

### V. CHANGES IN THE ENDOTHELIAL CELLS OF THE LYMPH NODES AND LIVER IN SPLENECTOMIZED ANIMALS RECEIVING HEMOLYTIC SERUM.\*

BY RICHARD M. PEARCE, M.D., AND J. H. AUSTIN, M.D.

(From the John Herr Musser Department of Research Medicine of the University of Pennsylvania, Philadelphia.)

#### PLATE 77.

In the course of our investigation<sup>1</sup> of the relation of the spleen to blood changes and to jaundice, routine histological examinations have been made. These have, for the most part, revealed only the well known lesions—liver necrosis, the degenerations occurring in epithelial organs, exudative lesions of the kidneys, and the general hemorrhages described by various workers<sup>2</sup> who have studied the

\* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, July 16, 1912.

<sup>1</sup> Pearce, R. M., Austin, J. H., and Krumbhaar, E. B., I. Reactions to Hemolytic Serum at Various Intervals after Splenectomy, *Jour. Exper. Med.*, 1912, xvi, 363; Pearce, R. M., Austin, J. H., and Eisenbrey, A. B., II. The Relation of Hemoglobinemia to Hemoglobinuria and Jaundice in Normal and Splenectomized Animals, *ibid.*, 375; Pearce, R. M., Austin, J. H., and Musser, J. H., Jr., III. The Changes in the Blood Following Splenectomy and Their Relation to the Production of Hemolytic Jaundice, *ibid.*, 758; Karsner, H. T., and Pearce, R. M., IV. A Study, by the Methods of Immunology, of the Increased Resistance of the Red Blood Corpuscles after Splenectomy, *ibid.*, 769.

<sup>2</sup> Kraus, R., Ueber Wirkungen der Hämolytine im Organismus, *Verhandl. d. deutsch. path. Gesellsch.*, 1903, v, 58.

Pearce, R. M., An Experimental Study of Nephrotoxins, *Univ. Penn. Med. Bull.*, 1903-04, xvi, 217; The Experimental Production of Liver Necrosis by Hemagglutinins, *Jour. Med. Research*, 1904, xii, 329; Concerning the Specificity of the Somatogenic Cytotoxins, *Jour. Med. Research*, 1904, xii, 1.

Fukuhara, Y., Zur Kenntnis der Wirkung der hämolytischen Gifte im Organismus, *Beitr. z. path. Anat. u. z. allg. Path.*, 1904, xxxv, 434.

Kraus, R., and Sternberg, C., Ueber Wirkungen der Hämolytine im Organismus, *Centralbl. f. Bakteriol., Orig.*, 1902, xxxii, 903.

action of hemolytic serum. The liver and lymph nodes, however, presented in a small group of animals changes which appear to be of significance in connection with the general problem of blood destruction in the absence of the spleen. The most striking of these changes, and the one that we shall describe, is the great increase in the phagocytic power of the endothelial cells of the liver and lymph nodes for red blood corpuscles.

It is not our intention to discuss the literature concerning the destruction of red cells. This has been well presented up to 1893 by Gabbi,<sup>3</sup> and up to 1901 by Hunter.<sup>4</sup> The more recent literature has added little either in fact or theory that is new. Out of the mass of contradictory statements there is uniformity of opinion on only two points: (1) that large endothelial cells of the spleen (the red blood corpuscle-carrying cells) have the power to engulf red blood cells; and (2) that the presence (in anemia and malaria) of blood pigment in the cells (Kupfer's cells) of the liver capillaries indicates that these cells play some part in the destruction of red blood cells. On the other hand, it is not generally admitted that the endothelial cells of the lymph nodes likewise have this power. That phagocytosis of red cells, wherever it occurs, leads ultimately to the freeing of hemoglobin which eventually reaches the liver and is transformed into bile pigment is the opinion of all who support the theory that this mechanism plays a part in the destruction of red blood cells. There is, however, no uniformity of opinion as to whether the hemoglobin is set free in the liver from red cells carried there by the phagocytes or whether it is set free by the phagocytes elsewhere and carried to the liver in another way.

It is only with one phase of the subject, the rôle of endothelial cells in engulfing red cells in the absence of the spleen, that we are concerned. Our hypothesis is that in the absence of the spleen the endothelial cells of the lymph nodes and liver compensate for the loss of similar cells of the spleen.

This possibility was first brought to our attention in the routine examination of tissues from splenectomized dogs which had received specific hemolytic immune serum. So striking were some of

<sup>3</sup> Gabbi, D., Ueber die normale Hämolyse mit besonderer Berücksichtigung der Hämolyse in der Milz, *Beitr. z. path. Anat. u. z. allg. Path.*, 1893, xiv, 351.

<sup>4</sup> Hunter, W., *Pernicious Anemia*, London, 1901.

the pictures, that we undertook, for the sake of control, the study of the liver and lymph nodes from a number of normal dogs, of normal dogs receiving hemolytic serum, and of dogs which had been splenectomized for various lengths of time, but which had not received hemolytic serum.

*Lymph Nodes.*—The lymph nodes studied have been, for the most part, the mesenteric, gastrohepatic, prevertebral, and bronchial. In the normal animal these have been examined more particularly for the frequency of mitosis, for the number of endothelial cells in the sinuses, and for the presence of cells containing red blood corpuscles. Careful study of nodes from five normal animals showed that mitotic figures are found only after prolonged search and are usually limited to the follicles. The number of endothelial cells varies, but usually is not great, and they never occur in large masses in the sinuses. These cells, however, not infrequently contain old blood pigment, and occasionally a cell may be seen containing one or two red blood corpuscles.

The lymph nodes of five animals splenectomized for 3, 4 (2), 39, and 84 days and not subjected to the action of hemolytic serum have been studied in the same way. In two animals representing respectively 4 and 84 days, the lymph nodes differed in no way from the normal; in the other animals mitotic figures were abundant in the follicles and the endothelial cells in the sinuses were greatly increased in number. Prolonged search, however, failed to demonstrate mitotic figures in the latter cells, and although they occasionally contained one or two red blood corpuscles, this power of phagocytosis did not appear to be greater than in the non-splenectomized animal. The increase in number of endothelial cells was, however, very striking.

The literature of splenectomy offers little aid in determining the histological changes occurring in the lymph nodes after removal of the spleen. In the literature at our disposal no definite descriptions have been found except those of Warthin,<sup>5</sup> who found in

<sup>5</sup> Warthin, A. S., *The Changes Produced in the Hemolymph Glands of the Sheep and Goat by Splenectomy*, Contributions to Medical Research, Dedicated to Victor Clarence Vaughan, Ann Arbor, 1903, 216.

sheep and goats an increase in the phagocytic power of the endothelial cells for red blood corpuscles.<sup>6</sup> Gabbi,<sup>7</sup> who worked with the guinea pig, states that a transient increase of the red blood corpuscle-carrying cells may possibly occur in early periods after splenectomy, but that after three to six months they are no more abundant than in the normal animal.

The lymph nodes of five normal dogs which had received specific hemolytic immune serum and had died or been chloroformed after periods varying from twenty-four hours to nine days, showed the lesions usually described as common to various cytotoxic sera and especially to lymphotoxic sera.<sup>8</sup> These are edema, increase of polymorphonuclear leucocytes, focal areas of necrosis, abundant mitotic figures in the follicles, and slightly greater frequency of large endothelial cells capable of phagocytosis of red cells.

Of animals that had been splenectomized and had received hemolytic serum as well, twelve were available for histological examination. Of these, three showed no change in the lymph nodes and five showed a well marked increase in the number of endothelial cells in the sinuses, but no increase in power of phagocytosis. In the four remaining animals the sinuses contained a great number of large endothelial cells filled with red blood corpuscles. The analysis of these findings is somewhat difficult, as three factors must be considered: (1) the length of time after splenectomy; (2) the lapse of time between administration of hemolytic serum and the death of the animal; and (3) the degree of red cell destruction caused by the serum.

These factors are brought out in the following table.

\* Warthin also describes a new formation of hemolymph nodes. This we have not observed in any of the nineteen splenectomized dogs. This, however, is not surprising as the demonstration of hemolymph nodes in the dog is difficult, and, moreover, only three of our animals have survived splenectomy for more than four months.

<sup>6</sup> Gabbi, D., *loc. cit.*

<sup>8</sup> Flexner, S., *The Pathology of Lymphotoxic and Myelotoxic Intoxication, Univ. Penn. Med. Bull.*, 1902, xv, 287.

Bunting, C. H., *The Effects of Lymphotoxins and Myelotoxins on the Leucocytes of the Blood and on the Blood-Forming Organs, Univ. Penn. Med. Bull.*, 1903-04, xvi, 200.

Experiment No.	Period of splenectomy.	Period after serum.	Effect of serum.	Histology.
I	3 dys.	36 hrs.	Hemoglobinuria	Extreme phagocytosis of red cells.
II	10 mos.	36 hrs.	Severe jaundice	Well marked phagocytosis of red cells.
III	9½ mos.	18 hrs.	No record	Well marked phagocytosis of red cells.
IV	7½ mos.	48 hrs.	Hemoglobinuria	Moderate phagocytosis of red cells.
V	15 dys.	3 dys.	Hemoglobinuria	Negative.
VI	27 dys.	3 dys.	Hemoglobinuria	Proliferation of endothelial cells.
VII	33 dys.	4 dys.	Jaundice	Proliferation of endothelial cells.
VIII	65 dys.	8 dys.	Hemoglobinuria	Proliferation of endothelial cells.
IX	6 dys.	9 dys.	Hemoglobinuria	Negative.
X	3 dys.	9 dys.	No hemoglobinuria or jaundice	Negative.
XI	103 dys.	10 dys.	No hemoglobinuria (spontaneous jaundice)	Proliferation of endothelial cells.
XII	25 dys.	15 dys.	Jaundice	Proliferation of endothelial cells.

From this analysis it is seen that the proliferation of the endothelial cells did not occur in the animals (V, IX, and X) splenectomized for periods of from three to fifteen days, but was evident in five (VI, VII, VIII, XI, and XII) in which the time elapsing since splenectomy was 27 to 103 days. On the other hand, the lymph nodes of these animals did not present evidence of increased phagocytosis of red cells. Whether this was due to the period which had elapsed (three to fifteen days) since injection of serum cannot be determined, but this was probably the case. Certainly it was not due to failure of hemolysis, for at least two of these animals (VI and VIII) presented evidence of extreme blood destruction. That the period of time elapsing may be an important factor is shown by the fact that all animals (four) presenting evidence of extensive phagocytosis of red cells represent periods of eighteen to forty-eight hours after injection of the serum. In the absence of exact knowledge of the length of time necessary for the destruction of red cells by phagocytic endothelial cells, it is useless to offer surmises, but one cannot escape the fact that in this investigation all evidence of active phagocytosis is seen in animals dying within forty-eight hours. It is possible therefore that the destruction of red cells by phagocytosis may be completed within forty-eight hours and this view is supported by the frequency with which pigment is found in the lymph nodes at later periods.

It is also evident that the time elapsing since splenectomy bears no relation to the occurrence of phagocytosis of red cells, for the most marked example of the latter was seen in a dog dying three days after splenectomy, while moderate and well marked phagocytosis occurred likewise after  $7\frac{1}{2}$ ,  $9\frac{1}{2}$ , and 10 months.

*Liver.*—Examination of the stellate endothelial cells (Kupfer's cells) of the liver has been rendered difficult on account of the intense congestion and abundant necroses which occur after the administration of hemolytic immune serum. For this reason we have not always been able to correlate the evidence of phagocytosis in the liver with the lesion described in the lymph nodes during the early (forty-eight hour) period. Definite evidence, however, of phagocytosis has been found in four animals, representing periods of 1, 2, 8, and 9 days after the administration of serum, and representing, respectively, periods of 3 days,  $7\frac{1}{2}$  months, 65 days, and 6 days after splenectomy. Also in a fifth animal, twenty-five days after splenectomy, and fifteen days after the administration of the serum, the cells of the capillaries contained small balls of yellow pigment, apparently representing altered hemoglobin.

As controls we have examined the livers of several normal dogs and of nine splenectomized dogs not receiving serum, but without finding evidence of phagocytosis on the part of the cells of the liver capillaries, or of proliferation of these cells. The splenectomies in this series represented periods of from three to eighty-four days, five under ten days and three over twenty days.

Likewise we have examined the livers of nine normal dogs receiving hemolytic serum. In two of these the endothelial cells appeared to be increased somewhat in number, and no undoubted evidence of phagocytosis could be obtained. All other livers examined showed no changes in the cells of the capillaries.

The details of some of these observations follow:

#### PHAGOCYTOSIS OF RED BLOOD CELLS BY STELLATE CELLS OF THE LIVER.

*Dog 32.*—Splenectomy was performed under ether anesthesia on July 19, 1911; spontaneous jaundice was noted on March 8, 1912, and specific hemolytic immune serum injected intravenously on this date. Hemolysis resulted. The red cells dropped within three hours from 6,120,000 to 5,200,000 per cubic millimeter, and the hemoglobin, after twenty hours, to 42 per cent. Death occurred after forty-eight hours.

*Histology.*—The liver cells are pale, granular, stain poorly, and present here and there small areas of focal necrosis. The capillaries are dilated and contain much granular material and, as seen by the low power, numerous isolated round and oval clumps of red blood corpuscles. By higher power of the microscope these clumps of red cells are found to be, in large part, within endothelial cells (figure 4). Some of the red cells stain well with eosin, others appear as shadows. Other endothelial cells are seen which contain mere fragments of red cells or masses of granular, yellow pigment, or large, yellow hyaline balls of apparently fused, red cells. Attempts to demonstrate similar phagocytic cells in the large vessels of the liver and of other organs failed; they were present, however, in the sinuses of the lymph nodes.

#### PHAGOCYTOSIS OF RED CELLS BY ENDOTHELIAL CELLS OF THE LYMPH NODES.

*Dog 34.*—Splenectomy was performed under ether anesthesia on March 11, and hemolytic serum administered intravenously on March 14. Death occurred on March 15 after reduction of red cells to 1,960,000 and hemoglobin to 57 per cent. Hemoglobinuria was marked.

*Histology.*—A mesenteric lymph node shows hemorrhage, edema, and extensive infiltration with polymorphonuclear leucocytes. The sinuses, both peripheral and central, are closely packed with large, pale, endothelial cells, nearly all of which contain red blood cells, a single high power field showing thirty to forty phagocytic cells (figures 1 and 2). The number of engulfed red cells varies but is usually large, ten to twenty not infrequently being found in a single cell. In many endothelial cells, on the other hand, the red cells have fused to form large, round, or oval hyaline masses still staining deeply with eosin. Between the phagocytic cells is much granular, eosin-staining material suggesting disintegrated red cells, mingled with serum, through which run irregular threads of fibrin. Here and there in the follicles are small areas of necrosis. Moderate leucocytic infiltration is seen throughout the section. Phagocytic cells cannot be demonstrated in the blood-vessels or in a tangle of lymphatic vessels present at one side of the node.

Other lymph nodes (gastrohepatic, prevertebral, and bronchial) present the same lesions.

The liver of this animal showed widespread necrosis but in the non-necrotic areas phagocytic endothelial cells are found in the capillaries (figure 3).

#### SUMMARY.

In a large proportion of dogs that have been splenectomized for periods of two weeks or more, one finds a great increase in the number of endothelial cells of the lymph nodes. In most splenectomized dogs that succumb to an injection of hemolytic immune serum within forty-eight hours, the sinuses of the lymph nodes contain large numbers of endothelial cells, phagocytic for red cells. This is not seen in normal dogs receiving hemolytic serum. Like-



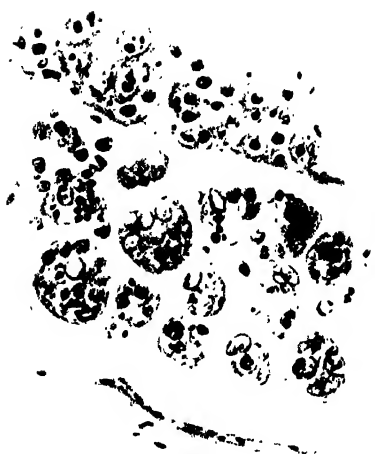
wise a similar power of phagocytosis is seen frequently in the stellate cells of the capillaries of the liver. Both in the lymph nodes and the liver these cells appear to be formed *in situ*; we find no evidence that they have been transported to these organs.

Such findings suggest the development of a compensatory function on the part of the lymph nodes and possibly of the liver. Normally the spleen contains cells which have the power to engulf and presumably to destroy the red blood corpuscles. In certain pathological conditions this function is frequently greatly augmented and may sometimes be shared by the lymph nodes, for example, in typhoid fever, as was first clearly shown by Mallory.<sup>9</sup> Our observations suggest that in the absence of the spleen, this function of forming red blood corpuscle-phagocytizing cells, normally a minor activity of the lymph nodes, becomes highly developed in the latter organs, and that these cells, and the stellate cells of the liver, thus assume, in part at least, the function of destroying red blood corpuscles by phagocytosis.

In view of the somewhat limited material at our disposal, we offer this, not as definitely conclusive, but as evidence which, in connection with the work of others, is highly suggestive of the possibility of the lymph nodes assuming some of the function of the spleen.

Whether this activity of the endothelial cells of the lymph nodes and the liver has any bearing on the anemia that follows splenectomy and on the occurrence of spontaneous jaundice in the late periods after splenectomy, is not yet clear.

<sup>9</sup> Mallory, F. B., A Histological Study of Typhoid Fever, *Jour. Exper. Med.*, 1898, iii, 611.



1



5



2



4



EXPLANATION OF PLATE 77.

The drawings were made with the camera lucida and with a Spencer microscope, objective, 4 mm., ocular, 8.

Figures 1, 2, and 3 represent lesions in a dog that was splenectomized on March 11, 1912, received hemolytic serum on March 14, and died on March 15. The actual lapse of time was about thirty-six hours.

FIG. 1. Peripheral sinus of a mesenteric lymph node containing large numbers of endothelial cells filled with red blood corpuscles and occasionally also polymorphonuclear leucocytes.

FIG. 2. Similar cells in a central sinus of the same lymph node.

FIG. 3. A section of liver with two Kupfer cells containing red blood corpuscles.

FIG. 4. Similar to figure 3, but from a dog that was splenectomized on July 19, 1911, received hemolytic serum on March 8, 1912, and died after forty-eight hours.

## NITROGEN RETENTION IN THE BLOOD IN EXPERIMENTAL ACUTE NEPHRITIS OF THE CAT.\*

BY OTTO FOLIN, PH.D., HOWARD T. KARSNER, M.D.,  
AND W. DENIS, PH.D.

(From the Laboratory of Pathology (Phillips Fund) and the Laboratory of  
Biological Chemistry of the Harvard Medical School, Boston.)

The literature and problems of experimental nephritis have been recently considered by Pearce<sup>1</sup> and it is therefore unnecessary to repeat them at this time. Considerable work has been done on the subject of nitrogen metabolism in acute nephritis and somewhat variable results have been attained. The development of the colorimetric methods for nitrogen determinations in the blood<sup>2</sup> has opened up new fields for work, and the object of the present study is to discover from the standpoint of blood analysis the possible retention of nitrogen in experimentally produced nephritis.

The types of nephritis selected for the study are those produced by uranium nitrate, by potassium chromate, and by cantharidin. This study must therefore be looked upon as preliminary to a more extensive series of other types of acute nephritis. A brief description of the type of nephritis produced in the cat by the poisons mentioned precedes each group of experiments; a more extensive study of the anatomical lesions found in the cat will be made the subject of a separate report.

The cats were kept under observation for from a week to ten days preceding the experiment. The animals were fed at 9 A. M. and bled at 3 P. M. The diet, a meat diet, was regulated so as to keep the weight constant, and frequent examinations of the urine were made in order to be sure that the animal had normal kidneys. All injections of the poisons were subcutaneous and the blood

\* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, July 31, 1912.

<sup>1</sup> Pearce, R. M., The Problems of Experimental Nephritis, Harvey Lectures, 1909-10, Philadelphia and London, 1910, 17; also in *Arch. Int. Med.*, 1910, v, 133.

<sup>2</sup> Folin, O., and Denis, W., Protein Metabolism from the Standpoint of Blood and Tissue Analysis, *Jour. Biol. Chem.*, 1912, xi, 164; New Methods for the Determination of Total Non-Protein Nitrogen, Urea and Ammonia in Blood, *Jour. Biol. Chem.*, 1912, xi, 527.

was obtained by aseptic puncture of the heart. Heart puncture was found to be satisfactory unless an extremely large needle was used. The blood was measured in volumetric pipettes. Five c.c. were withdrawn from the larger cats, 2 c.c. from the smaller animals, the amount being constant throughout each experiment. Further details of the technique are described in the second paper of Folin and Denis.<sup>2</sup>

Albuminuria was routinely tested for both by the heat and acid and by the nitric acid contact methods. The examination for casts was made in centrifuged specimens of urine, but frequently was unsatisfactory because of the large amounts of fat in the urine and the rich crystalline sediment.

#### URANIUM NEPHRITIS.

The most marked change histologically is in the epithelium, where cloudy swelling is found to be very extensive, in the later stages going on to complete necrosis. The glomerular tufts show moderate intercapillary proliferation which in later stages is obscured by the pushing back into the subcapsular space of epithelium of the type found in the proximal convoluted tubules.

Four cats were used as follows:

*Cat 1.*—Weight 3,550 gm. Given 0.002 gm. of uranium nitrate subcutaneously. Bled on the 3d day. Killed on the 3d day. Slight albuminuria on the 2d and 3d days. Few granular casts.

Time of bleeding.	Non-protein nitrogen in mg.	Urea nitrogen in mg.
Before injection.	32 <sup>4</sup>	20
48 hrs. after injection.	41	• 21

*Cat 2.*—Weight 1,700 gm. Given 0.0008 gm. of uranium nitrate subcutaneously. Bled on the 3d, 5th, 7th, and 9th days. Killed on the 9th day. Marked albuminuria up to the 6th day, then slight until termination. Few granular casts in earlier days.

Time of bleeding.	Non-protein nitrogen in mg.	Urea nitrogen in mg.
Before injection.	37	20
48 hrs. after injection.	50	27
96 hrs. after injection.	55	32
168 hrs. after injection.	44	19
216 hrs. after injection.	44	20

*Cat 3.*—Weight 4,000 gm. Given 0.002 gm. of uranium nitrate subcutaneously. Bled on the 2d, 6th, and 8th days. Found dead in cage on the 9th day (hemo-pericardium). Marked albuminuria in the earlier days remaining considerable to the end of the experiment.

<sup>2</sup> Folin, O., and Denis, W., *loc. cit.*

<sup>4</sup> The figures represent milligrams of nitrogen in 100 gm. of blood.

*Nitrogen Retention in the Blood.*

Time of bleeding.	Non-protein nitrogen in mg.	Urea nitrogen in mg.
Before injection.	31	21
72 hrs. after injection.	46	26
168 hrs. after injection.	100	36
216 hrs. after injection.	82	38

*Cat 4.*—Weight 1,540 gm. Given 0.0008 gm. of uranium nitrate subcutaneously. Bled on the 4th, 6th, 8th, and 10th days. Allowed to live. Albuminuria marked until the 8th day, very slight thereafter, disappearing on the 14th day. Hyaline and granular casts persisted until the 20th day.

Time of bleeding.	Non-protein nitrogen in mg.	Urea nitrogen in mg.
Before injection.	37	22
72 hrs. after injection.	55	30
144 hrs. after injection.	50	25
192 hrs. after injection.	42	20
240 hrs. after injection.	37	22

The doses of uranium nitrate employed were sufficient to produce a well marked nephritis anatomically, most severe in the case of cat 3. This cat also showed the most marked albuminuria and the highest nitrogen content in the blood after injection. The dose of uranium was proportionately very little larger than that given cat 1, but the process evidently was more severe both anatomically and functionally. It can fairly be said that the lesion produced by the injection of uranium nitrate results in the accumulation of non-protein and urea nitrogen in the blood much in excess of the normal amount, and as far as these experiments go, roughly in accordance with the degree of albuminuria and anatomical lesions. In all cases the retention appeared within 24 hours, persisted for as long as 216 hours, and in one case returned to normal after 240 hours.

**CHROMATE NEPHRITIS.**

The kidney lesion is almost purely tubular in type, the epithelium showing marked cloudy swelling and the appearance of hyaline droplets, the latter particularly in the distal convoluted tubules. The glomeruli occasionally show slight leucocytic infiltration or even early intercapillary proliferation, but for the most part are entirely free of involvement other than congestion.

*Cat 5.*—Weight 5,450 gm. Given 0.010 gm. of potassium chromate subcutaneously. Bled on the 2d and 4th days. Albuminuria slight from the 2d day to

the end of the experiment. Hyaline, granular, and fatty casts on the 2d day. Killed on the 4th day.

Time of bleeding.	Non-protein nitrogen in mg.	Urea nitrogen in mg.
Before injection.	40	19
24 hrs. after injection.	48	23
72 hrs. after injection.	38	24

*Cat 6.*—Weight 3,980 gm. Given 0.015 gm. of potassium chromate subcutaneously. Bled on the 2d, 4th, 6th, 8th, and 10th days. Albuminuria on the 2d day becoming progressively worse. Refused to eat after the 8th day. No casts at any time, but much epithelium and granular material in sediment. Killed on the 10th day.

Time of bleeding.	Non-protein nitrogen in mg.	Urea nitrogen in mg.
Before injection (1 week).	58	34
24 hrs. after injection.	40	22
72 hrs. after injection.	56	38
120 hrs. after injection.	60	40
168 hrs. after injection.	68	44
216 hrs. after injection.	44	30

*Cat 7.*—Weight 2,250 gm. Given 0.010 gm. of potassium chromate subcutaneously. Bled on the 3d, 5th, and 7th days. After bleeding on the 7th day, a second dose of 0.020 gm. of potassium chromate was given, and bleedings were continued on the 9th, 11th, and 14th days. Albuminuria did not appear until the 3d day and was slight until after the second chromate injection, becoming marked on the 8th, 9th, 10th, and 11th days, but disappearing on the 14th day. Casts did not appear until after the second injection, a few granular casts being found on the 8th day.

Time of bleeding.	Non-protein nitrogen in mg.	Urea nitrogen in mg.
Before injection.	40	28
48 hrs. after injection.	45	23
96 hrs. after injection.	46	34
144 hrs. after injection.	50	36
192 hrs. after injection.	58	38
216 hrs. after injection.	50	36
312 hrs. after injection.	54	34

*Cat 8.*—Weight 1,620 gm. Given 0.015 gm. of potassium chromate subcutaneously. Bled on the 2d day. Slight albuminuria on the 2d day. Killed on the 2d day.

Time of bleeding.	Non-protein nitrogen in mg.	Urea nitrogen in mg.
Before injection.	56	36
24 hrs. after injection.	55	35

The accumulation of nitrogen in the cats injected with potassium chromate is obviously less marked than in the uranium animals.



In cats 6 and 7 the accumulation appeared to be progressive, reaching its height several days after injection. In the case of cat 7 a second injection during the course of the disease appeared to increase the blood nitrogen to a considerable degree, more than would have been expected in the natural course of events if one might judge from cat 6. The degree of retention is apparently not at all proportional to the dose of drug employed but is roughly in accordance with the degree of albuminuria. The return to normal appeared in a severe case (cat 6) after 216 hours, and in a less severe case after 74 hours.

#### CANTHARIDIN NEPHRITIS.

Both glomeruli and epithelium show extensive lesions. The glomeruli show well marked intercapillary and intracapillary proliferation and moderate leucocytic infiltration. The lesion of the glomerular tuft is more marked than in either of the other forms studied. The tubular epithelium shows well marked cloudy swelling and the formation of numerous hyaline granules in the cells,—a change almost identical with that seen in chromate nephritis. Histologically the cantharidin lesion represents the chromate lesion with a superadded glomerular lesion of considerable severity.

*Cat 9.*—Weight 3,170 gm. Given 0.001 gm. of cantharidin in acetic ether subcutaneously. Bled 6 hours after injection and again on the 6th day. Killed on the 6th day. No albuminuria at 6 hours, moderate albuminuria at 48 hours and to the end of the experiment. No casts.

Time of bleeding.	Non-protein nitrogen in mg.	Urea nitrogen in mg.
Before injection.	36	24
6 hrs. after injection.	36	24
126 hrs. after injection.	40	28

*Cat 10.*—Weight 3,040 gm. Given 0.001 gm. of cantharidin in acetic ether subcutaneously. Bled 12 hours after injection and again on the 6th day. Slight albuminuria at 12 hours, disappearing before the 6th day.

Time of bleeding.	Non-protein nitrogen in mg.	Urea nitrogen in mg.
Before injection.	36	24
12 hrs. after injection.	45	32
132 hrs. after injection.	40	28

*Cat 11.*—Weight 3,700 gm. Given 0.001 gm. of cantharidin in acetic ether subcutaneously. Bled on the 3d day and then killed. Slight albuminuria.

Time of bleeding.	Non-protein nitrogen in mg.	Urea nitrogen in mg.
Before injection.	36	24
48 hrs. after injection.	41	26

*Cat 12.*—Weight 2,870 gm. Given 0.001 gm. of cantharidin in acetic ether subcutaneously. Bled on the 2d, 4th, and 7th days. Killed on the 7th day. Albuminuria on the 2d day, disappearing before the end of the experiment.

Time of bleeding.	Non-protein nitrogen in mg.	Urea nitrogen in mg.
Before injection.	36	22
24 hrs. after injection.	45	33
72 hrs. after injection.	41	26
144 hrs. after injection.	42	26

*Cat 13.*—Weight 2,760 gm. Given 0.002 gm. of cantharidin in acetic ether subcutaneously. Bled on the 2d, 4th, and 6th days. Vomited on the 1st day and ate poorly throughout the experiment. Albuminuria marked to end of the experiment. Killed on the 6th day.

Time of bleeding.	Non-protein nitrogen in mg.	Urea nitrogen in mg.
Before injection.	36	20
24 hrs. after injection.	66	41
72 hrs. after injection.	44	23
120 hrs. after injection.	40	29

*Cat 14.*—Weight 2,700 gm. Given 0.001 gm. of cantharidin in acetic ether subcutaneously. Bled on the 3d, 5th, and 7th days. Vomited on the 1st day and ate poorly up to the 5th day. Marked albuminuria throughout the experiment. Killed on the 7th day.

Time of bleeding.	Non-prote n nitrogen in mg.	Urea nitrogen in mg.
Before injection.	46	28
48 hrs. after injection.	64	38
96 hrs. after injection.	44	26
144 hrs. after injection.	50	34

In the nephritis produced by cantharidin the nitrogen retention is marked in the more severe cases. Of all the animals used, those receiving cantharidin were the only ones showing gastro-intestinal disturbance. Vomiting frequently appeared within a few hours after the injection, and anorexia persisted in some cases for four or five days.

The accumulation of blood nitrogen is clearly evident in all the cases; it shows no dependence on the amount of cantharidin injected, but accords with the degree of general disturbance and albuminuria; there was no discernible ratio between the degree of

anatomical involvement and the nitrogen accumulation. The increased nitrogen content, not apparent at six hours, became clearly evident at twelve hours, was most marked at twenty-four hours, and tended gradually to subside.

#### SUMMARY.

It will be seen that uranium nephritis, which involves both tubules and glomeruli, the former more markedly than the latter, produces a marked accumulation of nitrogen in the blood. Chromate nephritis, which involves almost exclusively the tubules, produces only moderate retention of nitrogen. Cantharidin nephritis which involves both tubules and glomeruli, the latter more severely than does uranium, produces a marked accumulation of nitrogen, beginning early and persisting for a considerable period. The experiments were controlled by testing the blood of normal cats kept under the same conditions, these animals showing only slight variations from day to day. This general statement is in accordance with the physiological classification of these nephritides except that the retention in uranium occurs at an early stage, where, according to the physiological studies of Schlayer and his associates,<sup>5</sup> and of Pearce, Hill, and Eisenbrey,<sup>6</sup> the vascular changes have not as yet appeared. It must be noticed that in the three types of nephritis that form the subject of this investigation, anatomical study shows the glomerulus to be distinctly involved in the two forms where accumulation of nitrogen in the blood is most marked, a condition indicating that although almost pure tubular involvement produces only moderate accumulation, the additional involvement of the glomerulus is extremely important in leading to a retention of nitrogenous waste products.

<sup>5</sup> Schlayer and Hedinger, *Experimentelle Studien über toxische Nephritis, Deutsch. Arch. f. klin. Med.*, 1907, xc, 1; Takayasu, R., *Ueber die Beziehungen zwischen anatomischen Glomerulusveränderungen und Nierenfunktion bei experimentellen Nephritiden*, *idem*, 1907, xcii, 127; Schlayer and Takayasu, R., *Untersuchungen über die Funktion kranker Nieren, München. med. Wchnschr.*, 1909, lvi, 2201.

<sup>6</sup> Pearce, R. M., Hill, M. C., and Eisenbrey, A. B., *Experimental Acute Nephritis: The Vascular Reactions and the Elimination of Nitrogen, Jour. Exper. Med.*, 1910, xii, 196.

The accumulation of non-protein nitrogen in the blood and tissues is not large when compared with the total intake or elimination of nitrogen, and consequently it is practically impossible by means of ordinary nitrogen equilibrium experiments to demonstrate the fact of the retention, to say nothing of determining the degree of accumulation of waste products accompanying nephritis. That both can be demonstrated by the method employed in this research is clearly shown by the figures recorded above.

## EXPERIMENTS ON INSECT TRANSMISSION OF THE VIRUS OF POLIOMYELITIS.\*

BY C. W. HOWARD AND PAUL F. CLARK.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

In considering the epidemiology of epidemic poliomyelitis we are confronted with a complex problem. Not only do we find cases which can be traced either through direct or indirect contact to other persons ill with the disease, but also we meet sporadic cases occurring over widely scattered areas and apparently well isolated from any acute source of contagion.

Both in human and in experimental poliomyelitis the virus has been repeatedly demonstrated in the tonsils and the secretions of the nasopharyngeal mucosa.<sup>1</sup> As the experimental disease can also be readily produced by swabbing the nasal mucosa with the virus, it has been held by many that the nasal mucosa is one, at least, of the sources of the virus in the outside world and also the means of its entrance to the body. The marked viability of the virus under adverse conditions, such as drying, low temperature, etc., and the fact demonstrated by Neustaedter and Thro<sup>2</sup> that the virus remains alive in dust, must also be considered as making for a fairly adequate explanation of the occurrence of cases in neighboring localities. More recently Kling, Wernstedt, and Pettersson<sup>3</sup> have brought forward experimental evidence that the nasal secretions may also be an important factor in the sporadic cases, as they claim to have found the virus in nasal washings from persons in contact with acute cases, as well as from the cases themselves.

\* Received for publication, October 10, 1912.

<sup>1</sup> Flexner, Simon, and Lewis, Paul A., *Jour. Am. Med. Assn.*, 1910, liv, 535; Osgood, R. B., and Lucas, W. P., *idem*, 1911, lvi, 495; Landsteiner, K., Levaditi, C., and Pastia, C., *Compt. rend. Acad. d. sc.*, 1911, clii, 1701.

<sup>2</sup> Neustaedter, M., and Thro, W. C., *N. Y. Med. Jour.*, 1911, xciv, 813.

<sup>3</sup> Kling, C., Wernstedt, W., and Pettersson, A., *Ztschr. f. Immunitätsforsch., Orig.*, 1911-12, xii, 316, 657; 1912, xiv, 303.

The fact that the seasonal incidence of the disease corresponds in general with the annual wave of insect life still suggests, however, the possibility that some insect may play a part in the transmission of this disease.

We have carried out experiments with several insects that are in close relation to human beings with reference to their ability to carry the poliomyelitic virus. Two types of insects come at once into consideration. One inhabits man's surroundings and acts as a mere contaminator of objects with which he is in intimate relation. These insects do not bite and are not to be viewed as possible active inoculators of the virus. An example of this group is the common house fly, *Musca domestica*. The other type comprises some of the biting insects. Among these are species that possess limited means of locomotion, such as the bedbug (*Cimex lectularius*) and lice (*Pediculus capitis* and *Pediculus vestimenti*), and others, such as the different mosquitos which have comparatively a much greater power of locomotion. These may conceivably act as active inoculators.

The domestic fly is by reason of its habits readily subject to gross contamination provided it can find access to infectious material. The existence of the poliomyelitic virus in the secretions of the nose and throat and also in the discharges from the intestine (Kling, Wernstedt, and Pettersson,<sup>4</sup> and Flexner, Clark, and Dochez<sup>5</sup>) makes such access possible. Whether the contamination ever occurs in nature is not known.

Our experiments were, in general, performed in two ways. In the first, insects were permitted to feed directly on infected cord or upon materials containing a suspension of infected cord. After the removal of the infected materials, the insects were placed in a fresh receptacle and after varying periods of time were killed and used whole or in part for the preparation of bacteria-free Berkefeld filtrates that were inoculated into monkeys. In this way we sought to determine whether the poliomyelitic virus survived either within the bodies of the insects or upon their external parts. In the

<sup>4</sup> Kling, C., Wernstedt, W., and Pettersson, A., *loc. cit.*

<sup>5</sup> Flexner, Simon, Clark, Paul F., and Dochez, A. R., *Jour. Am. Med. Assn.*, 1912, lix, 273.

second group, biting insects were made to feed upon monkeys inoculated with the virus of poliomyelitis. It has been shown by Flexner and Lewis<sup>6</sup> that the virus is present in the blood stream of infected monkeys, but only in rather minute quantities. The dilution is so great that twenty cubic centimeters or more of the blood from a recently paralyzed monkey are usually necessary to produce the disease in a normal animal. On account of this high dilution, it is especially valuable to determine whether the virus may be taken up by blood-sucking insects in sufficient quantity to produce infection when a filtrate of their bodies is employed for inoculating healthy monkeys. This experiment was carried out with bedbugs and lice.

#### EXPERIMENTS WITH FLIES.<sup>7</sup>

The flies used were the ordinary species, *Musca domestica*, bred in the laboratory. The technique of the experiment has been described above. After being allowed to feed on infected cord, the flies were placed in fresh receptacles from which certain numbers were removed at intervals, killed with ether, ground up with sand and physiological salt solution, and passed through a Berkefeld filter. The filtrate was injected as usual into the brain of *Macacus rhesus* monkeys. Protocols A and B show that the flies retained the virus either in or on their bodies for at least twenty-four and forty-eight hours respectively.

In order to determine the presence or absence of the virus actually within the bodies of the flies, the viscera of certain flies that had fed on infected cord were removed with aseptic precautions. Protocol C gives the history of such an experiment proving that the virus may remain alive in the body of the fly for at least six hours after ingestion.

*Protocol A.*—May 2, 1911. Injected a *Macacus rhesus* monkey intracerebrally with 3 c.c. of filtrate from the bodies of 7 flies which had had an opportunity to feed on infected cord for 5 hours and had then lived in clean surroundings for 24 hours before they were killed. The monkey recovered from the ether anesthesia and was apparently normal.

May 8. The monkey shows slight weakness.

<sup>6</sup> Flexner, Simon, and Lewis, Paul A., *Jour. Exper. Med.*, 1910, xii, 227.

<sup>7</sup> Flexner, Simon, and Clark, Paul F., *Jour. Am. Med. Assn.*, 1911, lvi, 1717.

May 9. Animal is nervous and irritable. Right arm is weak, left arm definitely paralyzed. During the day the paralysis affected the right arm.

May 10. The monkey is completely prostrate. The animal was killed with ether. At autopsy the cord showed the characteristic macroscopic lesions of experimental poliomyelitis. The microscopic sections were also typical.

*Protocol B.*—May 3, 1911. Injected a *Macacus rhesus* monkey intracerebrally with 4 c.c. of filtrate from the bodies of 10 flies which had fed on infected cord 48 hours previously. The monkey was apparently normal after recovery from ether anesthesia and remained well until May 13.

May 14. Animal is completely prostrate with paralysis of back muscles and all four extremities.

May 15. Animal was killed with ether. The autopsy showed characteristic lesions of experimental poliomyelitis in the spinal cord, and the sections, typical histological lesions.

*Protocol C.*—May 17, 1911. Injected a *Macacus rhesus* monkey intracerebrally with 5 c.c. of filtrate representing the viscera of 9 flies which had had an opportunity to feed on infected cord for about 18 hours. They were then removed to a clean receptacle for 6 hours, when they were etherized, and their legs, wings, and heads removed. The viscera were then taken out with sterile instruments, ground and filtered as usual, and injected as above. The monkey carries its head towards the left side after recovery from ether and seems somewhat dazed.

May 18. The monkey is active and apparently normal.

May 29. The animal shows a definite tremor and excitability and has the characteristic worried expression. The coat is fluffed up. Cerebrospinal fluid shows marked lymphocytosis and gives a positive test for globulin. 5.00 P. M. Arms very weak. Tremor is more marked. Animal gets up on perch with great difficulty.

May 30. Animal is prostrate. Legs and back muscles are partly but definitely paralyzed. Arms are completely flaccid.

May 31. Animal is moribund. Killed with ether. At autopsy the gross lesions in the cord were characteristic and the microscopic sections were also typical.

#### EXPERIMENTS WITH MOSQUITOS.

The mosquito experiments were performed in much the same way as were those with flies, except that the sticky, viscid nature of the incised cord caught the mosquitos as a piece of sticky fly-paper would, so a variation in the method of bringing them in contact with the virus was adopted. A mixture of defibrinated blood and a heavy suspension of cord from a recently paralyzed monkey was injected into washed figs, and these figs were suspended in the jars with the mosquitos. In this way the mosquitos apparently took the blood-virus mixture very well. After being fed on this



mixture for two days the mosquitos were placed in clean jars with non-injected figs and water and kept alive until wanted.

In two experiments the mosquitos used were the common *Culex pipiens*. In all the others the mosquitos were the salt marsh varieties, about 90 per cent. in each lot being *Culex sollicitans* and 10 per cent. *Culex cantator*.

Numbers of them were removed at intervals varying from three hours to twenty days after they had fed on the virus mixture. They were then killed, ground up, passed through a Berkefeld filter, and injected into the brain of a *Macacus rhesus* monkey. The purpose in allowing longer periods to elapse after the mosquitos had fed on the virus was to determine if possible whether there might be some biological transformation of the virus in the body of the mosquito as a secondary host. Fifteen monkeys were injected with mosquitos removed at different intervals and not one of these monkeys showed any symptoms of poliomyelitis. A control monkey inoculated with a minute portion of a fig injected with the blood-virus mixture came down typically with poliomyelitis.

Two illustrative protocols follow, showing the negative nature of the results, and also the protocol of the control animal (protocols D, E, and F).

*Protocol D.*—Aug. 12, 1911. Injected intracerebrally into a *Macacus rhesus* monkey 3.5 c.c. of filtrate representing the bodies of 20 mosquitos which had fed on the blood-virus mixture 24 hours previously. The animal remained normal and active.

*Protocol E.*—Sept. 16, 1911. Injected into the brain of a *Macacus rhesus* monkey 4 c.c. of filtrate representing the bodies of twelve mosquitos that had had an opportunity to feed on active poliomyelitis virus in figs for 2 days and had lived in clean surroundings for 20 days thereafter. The wings and legs were removed, the mosquitos ground up, filtered and injected as above. The monkey showed a slight weakness on the day following the injection but no other symptoms or paralysis.

*Protocol F.*—(Control of blood-virus mixture in figs.)

June 1, 1912. Injected into the brain of a *Macacus rhesus* monkey 3 c.c. of the following filtrate: On May 29 about 5 c.c. of a blood-virus mixture (2.5 c.c. of defibrinated horse blood and 2.5 c.c. of a 5 per cent. suspension of the cord from a recently paralyzed monkey) were injected into a fig weighing about 20 grams and allowed to remain at room temperature until June 1. A 5 per cent. suspension was then made of this fig and the material shaken, centrifugalized, and filtered. The filtrate was injected as above, thus introducing into the monkey filtrate which originally contained approximately 0.013 gm. of the cord.

June 6. Monkey somewhat inactive.

June 7. Slightly excitable. Arms and legs are weak, especially arms.

June 8. The arms, legs, and back muscles are paralyzed.

June 9. Found dead in the morning. At autopsy there were slight characteristic lesions in the cord and the microscopic sections were typical for experimental poliomyelitis.

#### EXPERIMENTS WITH LICE.

The experiments with *Pediculus vestimenti* and *Pediculus capitis* belong in the second general group; that is, the insects were allowed to suck the blood of several monkeys infected with epidemic poliomyelitis and then to remain alive for a number of days before they were killed, ground up in salt solution, filtered, and injected into monkeys. These insects proved to be difficult to manipulate, the mortality among them being very high. After their last feeding upon an infected monkey, it was found impossible to keep them alive for more than two to four days even though they were fed daily on a normal monkey. In only one experiment were we able to keep sufficient numbers of *Pediculus capitis* alive for as long as two days after their last feeding. The *Pediculi vestimenti* were used in five experiments in which they received from one to six feedings on monkeys with poliomyelitis, some in the preparalytic stage and others definitely paralyzed. The feedings were usually given either every day or on alternate days, so that in no case did any of the insects remain alive for more than thirteen days after the first feeding.

The results were entirely negative. The following are two illustrative protocols (protocols G and H).

*Protocol G.*—Dec. 19, 1911. Injected a *Macacus rhesus* monkey intracerebrally with 3 c.c. and intraperitoneally with 5 c.c. of filtrate representing in all 24 *Pediculi capitis* that had fed on a poliomyelitic monkey from Dec. 7 to Dec. 18. They were then made into a filtrate and injected as above on Dec. 19. The monkey showed no untoward symptoms or paralysis.

*Protocol H.*—Jan. 17, 1912. Injected a *Macacus rhesus* monkey intracerebrally with 2.25 c.c. of filtrate and intraperitoneally with 0.5 c.c. of filtrate representing 13 body lice which had fed on paralyzed monkeys from Jan. 9 to Jan. 13. On Jan. 15, the lice were fed on a normal monkey and on Jan. 16 they were ground up and passed through a Berkefeld filter. The monkey remained active and well.

## EXPERIMENTS WITH BEDBUGS.

The experiments with bedbugs (*Cimex lectularius*) also belong to the second group and were carried out as were those with the lice, except that the greater ease of manipulation made it possible to perform a more complete series.

The bedbugs were in all cases fed at least six times on infected monkeys in various stages of the experimental disease. More of the feedings were made on monkeys in the preparalytic stage than on those in a paralyzed condition because of the probably greater concentration of the virus in the blood stream during that period. It was found impossible to induce the bedbugs to feed until the previous meal had been digested, and, in the case of the immature forms, until the skin had been molted. Hence four or five days to a week passed between the successive feedings. After the last feeding on infected monkeys, periods ranging from a few hours to twenty-five days were allowed to elapse before the insects were ground up in salt solution and filtered preparatory to injection into a normal monkey. As in the case of the other insects this period between possible ingestion of the virus and the killing of the bedbugs was designed to answer the question of a possible life cycle in the body of the insect. Since many of the bedbugs were fed more than six times, some of them remained alive from seven to nine weeks after their first feeding.

In all, sixteen monkeys were injected with filtrates from the bodies of these bedbugs, and of this number only one showed evidence of infection with the virus of epidemic poliomyelitis. A repetition of the successful experiment, however, with the same time limits, etc., proved unsuccessful.

Two illustrative protocols are given.

*Protocol I.*—Dec. 18, 1911. Injected into the brain of a *Macacus rhesus* monkey 3 c.c. of filtrate, and intraperitoneally 7 c.c. of filtrate representing in all 20 bedbugs that had fed on poliomyelitic monkeys on the following dates: Nov. 8, 13, 15, 20, 29, and Dec. 5.

The filtrate was made on Dec. 15; thus an interval of 10 days occurred between the last feeding and the killing of the insects. The monkey showed no symptoms or paralysis.

*Protocol J.*—Nov. 24, 1911. Injected intracerebrally a *Macacus rhesus* with 2.5 c.c. of filtrate representing 10 bedbugs fed on infected monkeys on the

following dates: Oct. 23, 24, 27, Nov. 3, 8, 13, and 17. On Nov. 24, 7 days after the last feeding, the bedbugs were killed, filtered, and injected as above.

Nov. 30. Monkey somewhat slow. Arms are weak.

Dec. 1. Arms are partly paralyzed. The back and legs seem weak. Animal lies down most of the time.

Dec. 2. Animal moribund. Killed with ether. At autopsy the animal was found to have a severe tuberculosis of the spleen and left lung. The liver, mesenteric nodes, and right lung were also involved, but to a less degree. The right lung had only three or four tubercles. There were no definite macroscopic lesions in the cord. The microscopic sections, however, showed the typical lesions of experimental poliomyelitis.

A suspension of the glycerinated cord from this monkey was inoculated into another animal and this second animal came down typically, developing a definite paralysis on the sixth day. This animal was killed with ether. At the autopsy marked characteristic lesions were evident in the cord and the microscopic sections confirmed the result. The histological alterations in both spinal cords were characteristic of experimental poliomyelitis in the monkey and showed both infiltrative lesions about the blood-vessels in the interstitial substance and also degeneration and necrosis of ganglion cells and neurophagocytosis.

During the series of feeding experiments both with the lice and the bedbugs, the insects were sometimes allowed to feed on normal monkeys when no infected monkeys happened to be available at the time a feeding was necessary. Also during the varying intervals occurring after the last feeding on infected animals, normal animals were used in order to keep the insects alive. While this was not a definite attempt to study direct transmission by means of these insects, it is worthy of note that none of these normal animals so used ever showed the least symptom of poliomyelitis.

#### DISCUSSION.

The deductions from these experiments are clear. The domestic fly may become contaminated with the poliomyelitic virus which it may obtain in nature from infected discharges from the nose and throat or intestine. Because of the resistance of the virus to ordinary physical changes, especially when combined with mucous secretions, it can be transported in a living state on the surface of their bodies for two or more days, and because of the resistance it

displays to digestive secretions, within the esophagus and stomach for at least several hours. The survival in the gullet permits of the contamination of objects during the feeding process through the act of regurgitation which the fly performs in moistening and dissolving its food.

The flight of the house fly is considerable. Copeman, Howlett, and Merriman<sup>8</sup> found marked flies from one half to three quarters of a mile from the place of their liberation. On this account these potential passive contaminators are theoretically capable of carrying and depositing the virus at a considerable distance from the point at which the original contamination occurred. Through the ordinary active habits of the fly the virus may be transferred to persons or to things with which persons come into close relation, and by the death of the flies they may through disintegration liberate surviving virus that may possibly be converted into dust. The finding of the virus in the secretions of the nose, throat, and gastro-intestinal tract of human beings suggests that through the agency of such a passive contaminator as the fly, the virus may be taken into the bodies of human beings in which, conditions favoring, it may develop and cause infection.

Our experiments tend to exclude at least the species of mosquitos with which we worked as carriers of the virus. The pediculi also seem incapable of taking the virus out of the blood or of maintaining it in a living state within their bodies.

The experiments with the bedbug have a special interest. They were not designed either to ask or answer directly the question whether the bedbug acts as the source of infection in man. The question asked was whether they (or other biting insects) could take up the virus from the blood and maintain it for a period in a living state. When it is recalled that the virus exists in a highly dilute condition in the blood, this question assumes considerable importance. Although only a single successful experiment was secured, it is none the less clear in its meaning, which is that it is possible for a blood-sucking insect both to obtain living virus from the blood and to maintain it in a living state for at least seven days,

<sup>8</sup> Copeman, S. M., Howlett, F. M., and Merriman, G., *Reports of the Local Government Board on Public Health and Medical Subjects*, 1911, No. 53, 1.

at which time it can be inoculated successfully into the monkey and experimental poliomyelitis produced. Since, however, the experiment succeeds so exceptionally, the bedbug would seem not to be a carrier of the poliomyelitic virus in nature, although this possibility is not entirely excluded.

#### CONCLUSIONS.

The domestic fly (*Musca domestica*) can carry the virus of poliomyelitis in an active state for several days upon the surface of the body and for several hours within the gastro-intestinal tract.

Mosquitos (*Culex pipiens*, *Culex sollicitans*, and *Culex cantator*), in our experiments, have not taken up and maintained in a living state the virus from the spinal cord of monkeys.

Lice (*Pediculus capitis* and *Pediculus vestimenti*) have not taken the virus out of the blood of monkeys or maintained it in a living state.

The bedbug (*Cimex lectularius*) has taken the virus with the blood from infected monkeys and maintained it in a living state within the body for a period of seven days.

## THE EFFECTS OF SUBDURAL INJECTIONS OF LEUCOCYTES ON THE DEVELOPMENT AND COURSE OF EXPERIMENTAL TUBERCULOUS MENINGITIS.

### SECOND PAPER.\*

BY WILFRED H. MANWARING, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

In the first paper<sup>1</sup> it was shown that the injection of virulent tubercle bacilli into the basal meninges of dogs causes a tuberculous meningitis characterized by a latent period of from five to thirty days, depending upon the dosage and virulence of the injected organism, followed by a period of increasing paralysis and incoordination, ending almost invariably in death. It was further shown that the injection of living canine leucocytes into the meningeal cavities of these dogs, during the latent period of the disease, causes a delay in the development of the paralytic symptoms and a prolongation of the life of the treated animals:

This delay was most marked in dogs inoculated with small doses of the less virulent strains of tubercle bacilli. A number of the treated dogs so inoculated had developed no paralytic symptoms by the time the first paper went to press (five months), while the untreated control dogs had all developed paralytic symptoms after an incubation period of about four weeks, from which half of the control dogs had already died. The clinical history of these dogs is reviewed in figure 1.

This result was sufficiently encouraging to warrant a more extensive study of the effects of leucocytic injections, particularly a study of the effects of injections under conditions applicable to human medicine. It evidently would be quite difficult with human beings

\* Received for publication, September 25, 1912.

<sup>1</sup> Manwaring, W. H., *Jour. Exper. Med.*, 1912, xv, 1.

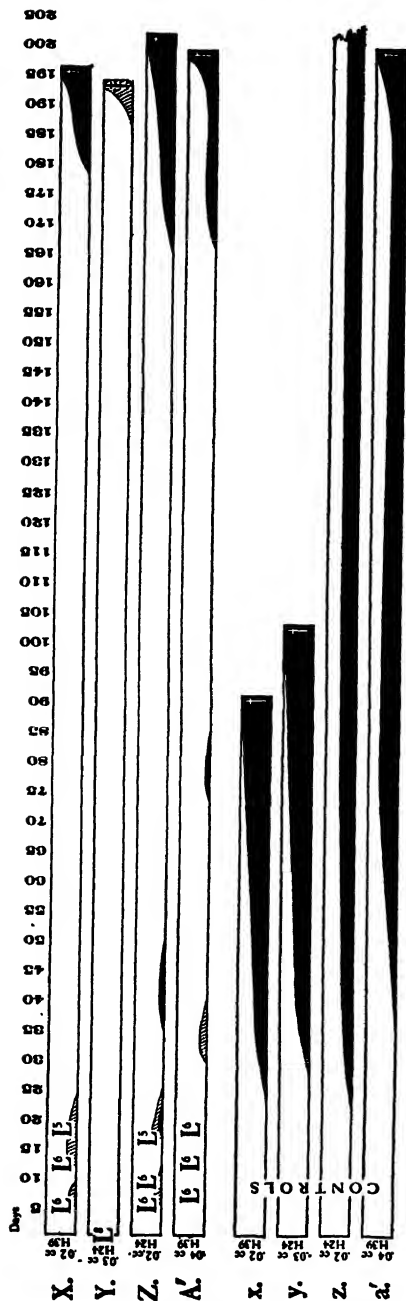


FIG. 1. EFFECTS OF SUBDURAL INJECTIONS OF HOMOLOGOUS LEUCOCYTES IN DOGS. Four dogs (X, Y, Z, A'), and controls (x, y, z, a'), were inoculated by the cranial-trephine method with small doses of tubercle bacilli, and given simultaneous or subsequent subdural injections with dog leucocytes (L). The dark areas show the development and the changes in the severity of the ataxic and paralytic symptoms. For further explanation of the figures see first paper.

The control dogs of this group all developed paralytic symptoms after an incubation period of from 25 to 35 days. The treated dogs were practically free from paralytic symptoms till the middle of the sixth month. One of the treated dogs (Y) died of distemper during the seventh month, and showed at autopsy no tuberculous lesion. The other treated dogs died toward the end of the seventh month, and showed at autopsy typical subdural tuberculosis.



to use homologous leucocytes as a therapeutic agent. Leucocytic therapy would be easy, however, if it were found possible to use leucocytes other than those of human origin. It was therefore desirable, as an initial step, to determine whether or not the same delay can be brought about in the development of the paralytic symptoms in inoculated dogs by the use of foreign leucocytes, and, if so, whether or not a similar delay can be produced by foreign leucocytes in animals whose susceptibility to tuberculosis more nearly approaches that of human beings. In the present paper an attempt has been made to answer these two questions.

Considerable work has already been done on the possible therapeutic uses of foreign leucocytes. Of particular interest in the present connection are the studies of Pettersson and his co-workers, who have tested the prophylactic and curative effects of subcutaneous and intraperitoneal injections of foreign leucocytes in a number of experimental infections, including experimental tuberculosis in the guinea pig.<sup>2</sup> Equally suggestive is the work of Hiss and his colleagues, who have studied the effects of injecting foreign leucocytic extracts.<sup>3</sup>

#### EXPERIMENTS ON DOGS.

*Toxicity of Foreign Leucocytes.*—The use of foreign leucocytes in meningeal infections in dogs is limited by their great toxicity when injected into the subdural spaces of these animals. Rabbit leucocytes,<sup>4</sup> injected in doses of from 0.7 to one cubic centimeter, apparently always cause death.<sup>5</sup> The dogs recover normally from the etherizations necessary for the injections, and show immediately after recovery no unusual symptoms. About two hours later there is beginning incoördination, rigidity, and respiratory distress, increasing to collapse by the fourth hour, and death by the sixth hour. An occasional dog survives till the twenty-fourth hour.

<sup>2</sup> Kling, C., *Ztschr. f. Immunitätsforsch., Orig.*, 1910, vii, 1.

<sup>3</sup> Hiss, P. H., Jr., and Zinsser, H., *Jour. Med. Research*, 1908, xiv, 321; 1909, xv, 245.

<sup>4</sup> For the method used in obtaining aseptic rabbit and horse leucocytes, see Manwaring, W. H., *Jour. Exper. Med.*, 1912, xvi, 250, 253.

<sup>5</sup> The inoculations and treatments of the dogs were made under morphin-ether anesthesia.

The cause of death in these injected dogs has not yet been fully determined. Rabbit leucocytes, killed by repeated freezing and thawing, cause no serious symptoms. The same is true of an aqueous extract of rabbit leucocytes. Autopsies on dogs dying after the injection of rabbit leucocytes show a hemorrhagic condition of the subdural tissues, suggesting the action of a hemorrhagin.

Horse leucocytes are less toxic for dogs. From one to three cubic centimeters can be injected into the cerebral meninges with comparatively few toxic symptoms. Approximately a quarter of the dogs so injected die within a few hours after the injection; but this mortality is not greater than the mortality previously noted after the injection of dog leucocytes. Horse leucocytes were therefore selected as the therapeutic agent to be tested with dogs.

The use of horse leucocytes, however, is limited by their increased toxicity on a second injection, or when injected into meninges the seat of a pathological lesion. The second injection of horse leucocytes into normal meninges, from five to seven days after the first injection, is apparently invariably fatal. A fatal result apparently always follows the injection of horse leucocytes from seven to ten days after the inoculation of the meninges with tubercle bacilli.

This limits the study of the effects of local injections of horse leucocytes in meningeal tuberculosis in dogs to a determination of the effects of a single injection of the leucocytes made simultaneously with the initial inoculation with tubercle bacilli.

*Effects of Simultaneous Inoculation with Horse Leucocytes and Tubercle Bacilli.*—The injection of from one to three cubic centimeters of horse leucocytes into the basal meninges of dogs, simultaneously with the inoculation of the meninges with minimal doses of tubercle bacilli, is apparently without effect upon the development and course of the subsequent tuberculous meningitis in the majority of the treated animals (figure 2). There is, however, in a certain number of the treated animals, a slight prolongation of the latent period, and in one of them (E) the subsequent paralytic symptoms have been unusually mild.

These effects, however, are very slight when compared with the remarkable prolongation of the latent period previously observed

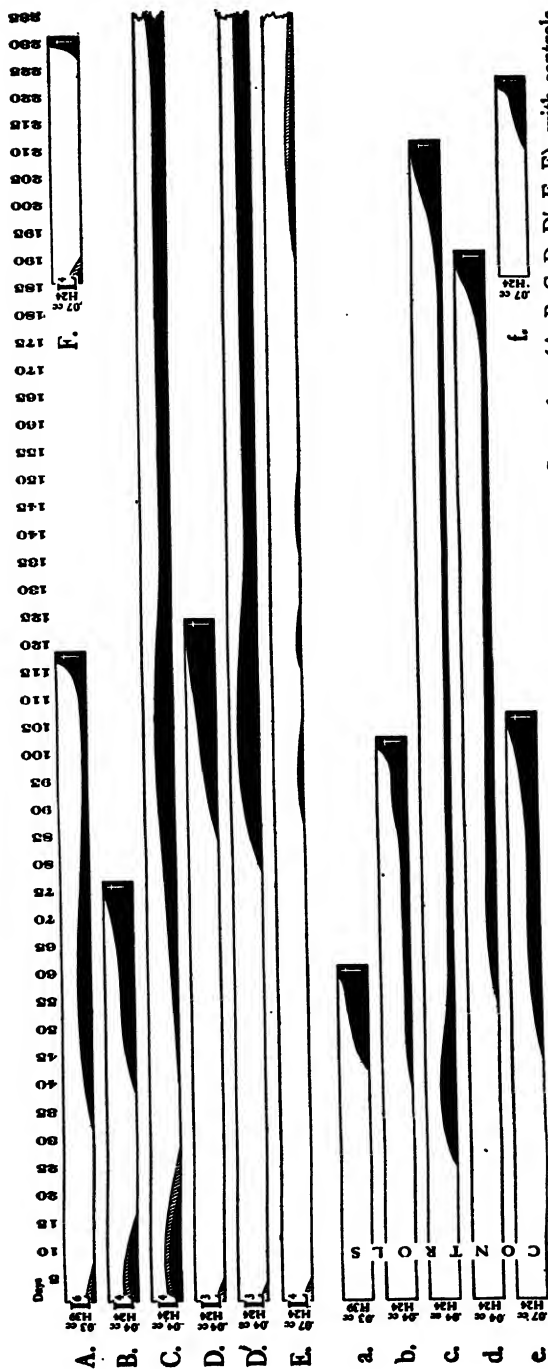


FIG. 2. SIMULTANEOUS INJECTIONS OF HORSE LEUCOCYTES AND TUBERCLE BACILLI IN DOGS. Seven dogs (A, B, C, D, D', E, F), with controls (a, b, c, d, e, f), were inoculated with small doses of tubercle bacilli, and given simultaneous injections of horse leucocytes (L). The cranial-trephine method was used in this group.

Three of the treated dogs (A, B, C) showed marked toxic and paralytic effects immediately after the injections, the symptoms lasting for from one to three weeks. These dogs all developed paralytic symptoms after an incubation period practically the same as that in the untreated controls (a, b, c). The other treated dogs (D, D', E, F) showed fewer immediate symptoms and all gave a distinct prolongation of the latent period. In one of the dogs (E) the subsequent paralytic symptoms have been unusually mild.

after the injection of dog leucocytes (figure 1). The average length of the latent period in the untreated control dogs of this group was thirty-eight days, that of the treated dogs, fifty-seven days, giving an average prolongation of but nineteen days, as compared with the five or six months' prolongation obtained with dog leucocytes.

The various samples of horse leucocytes used in these injections differed in their toxic effects. While one sample caused stupor and even a paralysis, lasting for from one to three weeks, a second sample, obtained under apparently identical conditions, caused only a slight quietness of the injected animal, which disappeared within a few days. It was noted that the prolongation of the latent period was produced only by the less toxic samples of the leucocytes.

The autopsy findings in the dogs dying after these inoculations and treatments are similar to those described in the previous paper. No constant differences have yet been found between the anatomical pictures in the treated and untreated dogs.

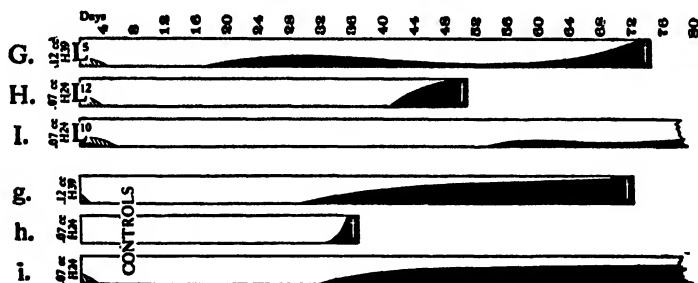


FIG. 3. SIMULTANEOUS INJECTIONS OF HORSE LEUCOCYTES AND TUBERCLE BACILLI IN DOGS. Three dogs (G, H, I), with controls (g, h, i), were inoculated and treated by the method of lumbar puncture. One of the treated dogs (I) has shown a slight prolongation of the latent period, and exceptionally mild subsequent paralytic symptoms.

Following an observation with monkeys, described in the latter part of this paper, an attempt was made to vary the technique of the inoculations by injecting the bacilli and leucocytes into the spinal meninges rather than into the basal meninges of the brain. Spinal inoculation can be accomplished in dogs by a modification of the ordinary method of lumbar puncture. The fore quarters of the

dog are elevated till its back is nearly vertical, and a long, slender, hypodermic needle is then passed obliquely through the lumbar enlargement of the cord, till the escape of cerebrospinal fluid shows that the needle has passed into the subdural space. In the subsequent clinical picture allowance has to be made for the occasional paralysis resulting from the necessary injury to the cord substance.

A small group of dogs in which the inoculations were made by the method of lumbar puncture is shown in figure 3. While the average length of the latent period is the same in the treated and untreated dogs of this group, one of the treated animals (I) has shown a slight prolongation of the latent period and a distinct lessening of the severity of the subsequent paralytic symptoms. This result, however, is no more striking than the result obtained above, by the routine method of cranial inoculation.

The results from the study of these two groups of dogs, however, were considered sufficiently encouraging to warrant an extension of the problem to other animals in which the study was not so greatly limited by the toxicity of the injected leucocytes. The work was, therefore, repeated on monkeys.

#### EXPERIMENTS ON MONKEYS.

*Toxicity of Foreign Leucocytes.*—Foreign leucocytes are apparently much less toxic for monkeys. This possibly depends upon the relatively greater capacity of the subdural space in monkeys, which tends to prevent any great or sudden increase in cerebrospinal pressure as a result of a local inflammatory reaction.

Rabbit leucocytes can be injected into the subdural space of monkeys in doses as high as one cubic centimeter, without the production of fatal symptoms. This injection can be made either into the spinal meninges by the ordinary method of lumbar puncture, or into the cerebral meninges by the cranial-trephine method previously described for dogs. The monkeys are usually somewhat quieter than normal for a day or two after the injection, and there are occasionally cranial tenderness and transient paralyses; but there is not the collapse and respiratory distress observed in dogs. Horse leucocytes are apparently even less toxic, and can be injected in doses of two cubic centimeters.

Monkeys, also, can be safely reinjected with foreign leucocytes after an interval of from five to seven days, without the production of unusually severe symptoms. If reinjected sooner than that, as after an interval of but three days, the monkeys often die with the symptoms of collapse and respiratory distress described above. No unusually severe symptoms are observed in monkeys if the leucocytes are injected into meninges previously inoculated with tubercle bacilli.

*Inoculation of the Meningeal Cavity with Tubercle Bacilli.*—In monkeys, inoculation of the meningeal cavity can be readily made by the method of lumbar puncture. The tuberculous meningitis produced by such inoculations gives a clinical picture similar to that observed in dogs. Following the inoculation there is a latent or incubation period of several weeks, during which time no characteristic symptoms are observed. In the later stages of this period the animal may be somewhat quieter than normal and may show a greater tendency to chatter or cry out when disturbed. This latent period is followed by a period of increasing paralysis and incoördination, ending in collapse and death. The paralysis after lumbar inoculation invariably begins in the hind legs.

The beginning of the paralysis is usually less easily detected in monkeys than in dogs, due to their great activity. In monkeys, as in dogs, the disease is apparently invariably fatal.

The autopsies on monkeys dying as a result of lumbar inoculation usually show a layer of diffuse tuberculous tissue filling the subdural space, with more or less necrosis and infiltration of the superficial portions of the spinal cord and nerve trunks. The most important finding for our present purpose, however, is the almost constant presence of extradural tuberculosis in these animals. Extending in some cases the entire length of the spinal cord, but generally confined to a few centimeters above and below the site of inoculation, are numerous miliary and conglomerate tubercles, which fill and often entirely replace the extradural spinal tissue. These extradural tubercles are usually not adherent to the dura, and evidently arise from a leakage of the infectious agent into the extradural tissue spaces through the dural puncture made at the time of the inoculation.

Since these extradural lesions cannot be reached by a therapeutic agent injected into the meningeal cavity, and since their presence alone would be sufficient to cause paralytic and ataxic symptoms, the constant presence of extradural tuberculosis in monkeys inoculated by the method of lumbar puncture renders inoculation and treatment by this method impracticable in the present investigation. The later inoculations and treatments herein reported were therefore made through a permanent wax-trephine opening in the skull, as described for dogs in the preceding paper.

The clinical picture in monkeys inoculated by the cranial method<sup>6</sup> is similar to that after inoculation by lumbar puncture. The initial paralyses are possibly less liable to be confined to the hind quarters, and occasionally death occurs even without the presence of a definite paralysis, there being only an increased quietness and stupor, with a slight spasticity of the neck muscles.

For our present purpose the most important autopsy finding in monkeys inoculated by the cranial method is the complete absence of extradural tuberculosis. The extradural tissues have been found to be free from tuberculosis in all monkeys inoculated by this method.

The distribution of the tuberculous lesion within the meningeal cavity, however, differs somewhat in these animals from the distribution after lumbar inoculation. Scattered over the surface of the brain are numerous tubercular foci, usually small, well marked tumor masses. The main lesion, however, in these monkeys, as in monkeys inoculated by lumbar puncture, is situated in and around the lower half of the spinal cord. Here the subdural space is usually completely filled with diffuse and conglomerate tuberculous tissue. It would appear as though most of the injected tubercle bacilli had gravitated to the lower levels of the cord, as a result of the constant upright position of the animals.

*Effects of Leucocytic Injections.*—In the earlier experiments a number of monkeys were inoculated and treated by the simpler method of lumbar puncture. Those of the earlier monkeys that received simultaneous or subsequent injections of horse leucocytes are shown in figure 4. All the treated monkeys of this group devel-

<sup>6</sup>The inoculations were made under ether anesthesia.

oped paralytic symptoms occasionally even after a shorter incubation period than the untreated controls. The average length of the incubation period in the treated monkeys of this group was shorter than that of the untreated controls, as was also the average duration of life.

This experiment, however, is not thoroughly conclusive as to the negative value of the injection of horse leucocytes, since there was with each member of this group a loss from the meningeal cavity of

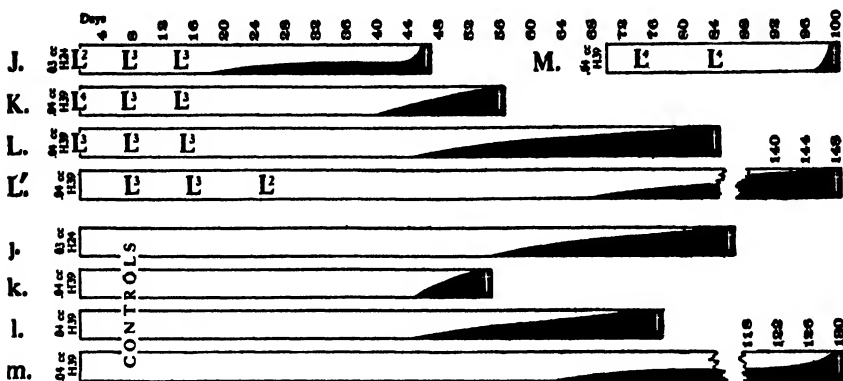


FIG. 4. EFFECTS OF HORSE LEUCOCYTES IN MONKEYS. Five monkeys (J, K, L, L', M), with controls (j, k, l, m), were inoculated by the method of lumbar puncture, and given simultaneous and subsequent injections of horse leucocytes. The number of cubic centimeters of a 30 per cent. suspension of leucocytes injected are shown by the small exponents.

Two of the treated monkeys (J, M) showed a distinct shortening of the latent period, and one monkey (L') a possible prolongation of that period. Autopsies showed extradural tuberculosis in all the members of this group.

part of the injected dose of tubercle bacilli, with the setting up of extradural tuberculosis.

A second group of monkeys, also inoculated by the method of lumbar puncture, was given simultaneous and subsequent injections with rabbit leucocytes (figure 5). In one of the treated monkeys of this group (O) there was a considerable prolongation of the latent period, and a second monkey (P), killed at the end of seven months, during which time it had shown no paralytic symptoms, was found on autopsy to be apparently free from tuberculosis. Whether this means an actual prevention of the development of tuberculosis



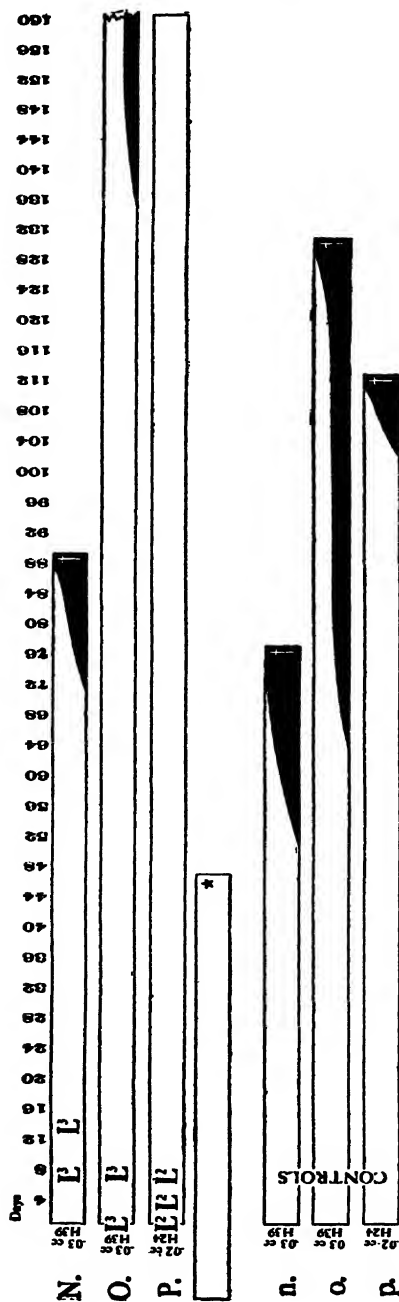


FIG. 5. EFFECTS OF RABBIT LEUCOCYTES IN MONKEYS. The monkeys (N, O, P), with controls (n, o, p), were inoculated by the method of lumbar puncture, and given simultaneous or subsequent injections with rabbit leucocytes. Two of the treated monkeys (O, P) showed a marked prolongation of the latent period, and one of them (P), killed at the end of seven months (\*), was found to be free from tuberculosis. The interpretation of the result in this group is complicated by the presence of extradural tuberculosis.

in this animal as a result of the injections of rabbit leucocytes, or merely that the monkey selected possessed an unusual resistance to tuberculosis, can be determined only by a repetition and extension of this work.

These earlier observations with rabbit leucocytes, uncertain as their interpretation may be, were sufficiently suggestive to warrant a study of the effects of rabbit leucocytes when injected under conditions that eliminate the confusion from extradural tuberculosis, and rule out the possible experimental error from individual variations in resistance. A study of the effects of injections of rabbit leucocytes by the cranial method was, therefore, begun.

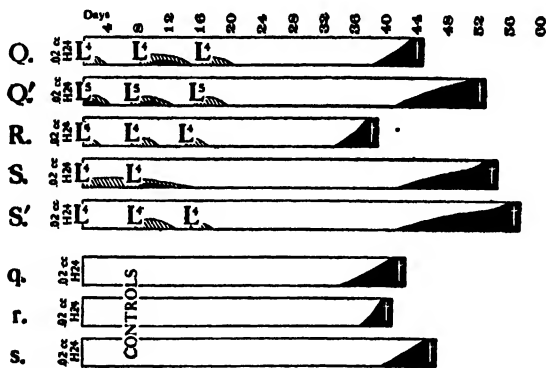


FIG. 6. EFFECTS OF RABBIT LEUCOCYTES IN MONKEYS. Five monkeys (Q, Q', R, S, S'), with controls (q, r, s), were inoculated and treated by the cranial-trephine method. There was practically no difference observed between the treated and untreated monkeys of this group. This negative result, however, is not necessarily final, due to the possibility of the monkeys of this group having been given an overdose of the infectious agent. All of the monkeys of this group were free from extradural lesions.

A group of monkeys, inoculated and treated by this method, is shown in figure 6. The five monkeys of this group receiving simultaneous and subsequent injections with rabbit leucocytes, all developed paralytic symptoms after practically the same incubation period as the three untreated controls. All the monkeys of this group died at practically the same time. The autopsies showed subdural tuberculosis in all the members of the group, the distribution of the lesions being practically the same in the treated and untreated animals.

In so far, however, as the dosage of the tubercle bacilli injected in this group was based upon the earlier experience with lumbar inoculations, in which there was a considerable loss of the infecting agents from the meningeal cavity, it is conceivable that all the monkeys of the group received an overdose of the infectious agent. It will be recalled that in the first paper the dogs receiving an overdose of tubercle bacilli showed no prolongation of the latent period on treatment with dog leucocytes. It was only when the dosage was reduced that a prolongation was observed. The probability, however, is against there having been such an overdose, since there was a long latent period with all the members of this group. The work with monkeys is being continued.

#### SUMMARY.

Rabbit leucocytes, injected into the basal meninges of dogs, in doses from 0.7 to one cubic centimeter apparently invariably cause death. Horse leucocytes, injected in the same amounts, cause death in about 25 per cent. of the dogs.

The injection of foreign leucocytes into the meninges of monkeys causes few if any symptoms.

The injection of from one to three cubic centimeters of horse leucocytes into the meningeal cavities of dogs, simultaneously with the inoculation of the meninges with tubercle bacilli, causes a slight delay in the development of the paralytic symptoms in about half the treated animals. This delay, however, is very slight when compared with the remarkable prolongation of the latent period previously observed after treatment with dog leucocytes.

The injection of foreign leucocytes into the meningeal cavities of monkeys has thus far given almost uniformly negative results. In one small group of monkeys, however, inoculated by the method of lumbar puncture, the injection of rabbit leucocytes has been associated with a prolongation of the latent period in one of the treated monkeys, and with a complete prevention of the subsequent tuberculosis in a second monkey.

## CULTIVATION OF TREPONEMA CALLIGYRUM (NEW SPECIES) FROM CONDYLOMATA OF MAN.\*

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

### PLATES 6 AND 7.

While engaged in the cultivation of various species of spirochætæ found in the oral cavity or about the lesions on the external genitals and the anus of human beings, I encountered on two occasions a spirochæta which morphologically occupies an intermediary position between *Treponema pallidum* and *Spirochæta refringens*. The material was taken in one instance from a condyloma latum in which both the pallidum and this organism were present. In another instance, it was found in a condyloma situated around the anus of a boy, nine years of age, in whom the existence of syphilis was finally ruled out by clinical and serological examinations.<sup>1</sup> In this case, the condylomatous tissue was excised and inoculated into the testicles of three rabbits by the usual method, but no lesion resulted during a period of four months. An emulsion of the tissue examined under the dark-field microscope showed some contaminating bacteria, but no other spiral organisms save the one under consideration. In the sections stained by the Levaditi silver method, the spirochætæ were found on the surface, but not within the tissue. No pallidum was detected. The spirochæta in question could hardly be mistaken for *Treponema pallidum* as it appeared decidedly thicker than the latter, although some specimens had quite deep and regular curves. The extremities were obtuse or sharply tapered, and some possessed delicate, finely curved projections at one or both ends. The movements were chiefly those of rotation around the axis, but there was also a change in the form of the curves. Neither

\* Received for publication, October 1, 1912.

<sup>1</sup> A medico-legal case.

a vibratory nor lateral movement such as is common with *Treponema macrodentium* occurred. The spirochætæ measured about 0.35 to 0.4 of a micron in width, and ranged from a few microns to twenty microns in length. The number of curves was determined by the length of the organism, and in typical specimens the distance between the curves was usually about 1.6 microns. The curves were not as closely set as in the pallidum. Compared with *Spirochæta refringens* it was somewhat thinner, more regular, and possessed more numerous curves and showed less motility. Stained with Giemsa's solution, it was red with a faint violet tint.

*Cultivation.*—Emulsions of the condylomatous tissue were used in both cases. The method employed consisted in inoculating a series of tubes containing a mixture of ascitic fluid (one part) and slightly alkaline agar (two parts) containing a piece of fresh sterile tissue at the bottom of each tube. The introduction of the emulsion was made with the aid of a capillary pipette connected with a syringe. After inoculation the tubes were covered with a layer of sterile paraffin oil and placed in a thermostat at 37° C. At the end of ten days, a hazy zone radiating from the central stab canal, which was now densely filled with the growth of bacteria, appeared along the canal up to three centimeters of the surface.

*Purification.*—After two weeks' growth some of the best tubes were selected and the organisms in the hazy zone were examined under the dark-field microscope. The colonies from the first case (syphilitic) revealed the presence of two distinct varieties of spirochætæ; namely, a thin, regularly and deeply curved organism, and a thick but also regularly curved one. The thin type was undoubtedly the pallidum and the thick one the spirochæta in question. The colonies developing in the tubes inoculated with the material from the second case (non-syphilitic) showed the presence of only one type of spirochæta which was in all respects identical with the form found in the original material.

I was able to separate these organisms from the bacteria.<sup>2</sup> While colonies of the syphilitic material contained both the pallidum and the second organism in the first generation, the pallidum type finally disappeared in subsequent subculture, thus leaving the thicker type

<sup>2</sup> Noguchi, H., *Jour. Exper. Med.*, 1912, xv, 90.

in pure condition. The purified culture of the second or non-syphilitic material yielded solely colonies of this organism.

*Properties of Pure Culture.*—When transplanted into the ascitic agar tissue medium, it develops so far in three or four days as to show hazy, somewhat opalescent colonies about the tissue. During the following several days the colonies gradually spread towards the upper part of the medium, thus rendering the latter unevenly filled with hazy opalescent colonies. At the end of four weeks the growth usually reaches the greatest height that it ever attains,—about two centimeters from the surface. The colonies are diffuse and usually somewhat more dense than those of *Treponema pallidum*. The organism grows well in fluid medium containing ascitic fluid when cultivated by the special method described for *Treponema pallidum*.<sup>3</sup> The growth of this spirochæta does not bring about any visible change in the medium and there is no perceptible odor produced. Like *Spirochæta refringens* it is capable of growing in ascitic agar without the addition of fresh sterile tissue, although the growth is far better in the presence of the tissue. The organism is a strict anaerobe. There is no growth at room temperature.

*Morphology.*—(Figures 1, 2, 6, 12, 13, and 14.) In solid cultures three or four days old the majority of the organisms are short and vigorously motile, while in cultures which have been growing for about two weeks they are longer and less active. In fluid medium long specimens as well as short ones are present from the beginning. The length as well as the width of the organisms is more variable in the fluid cultures.

The spirochæta in pure culture measures about 0.35 to 0.4 of a micron in width and 6 to 14 microns in length. Forms as short as 4 microns or as long as 30 microns, according to the age and nature of cultures, may occur. In old fluid cultures, individuals measuring in width as little as 0.3 or as much as 0.45 of a micron are apt to appear. The curves are very regular and quite deep. The distance between the curves is about 1.6<sup>4</sup> microns and the depth of a curve is about 1 to 1.5 microns. The apex of curves is not angular, but

<sup>3</sup> Noguchi, *Jour. Exper. Med.*, 1912, xvi, 211.

<sup>4</sup> The wave length of *Treponema pallidum* measures about 1.2 microns and that of *Treponema microdentium* about 1 micron.

more or less rounded. In the short specimens the curves are less constant and often obliterated through movements of the body. While the curves of less motile specimens remain unmodified during movement, those of vigorously active specimens change their form in an undulatory manner. A small number of individuals have shallower curves or become almost straight, especially near the middle portion of the body; but I regard these as the irregularities due to the abnormal effect of artificial cultivation. The body has a uniform width until toward the terminal portion which ends in a sharp point. In this respect it resembles *Spirochæta refringens* in pure culture, and not *Treponema macrodentium*, whose body gradually thins out towards the ends (figure 8).

The spirochæta shows often a delicate, regularly curved, rather stiff projection attached to one or both ends. The length of this appendage varies in different specimens, but usually measures about six microns. The presence or absence of this projection seems to depend greatly upon cultural conditions and the age of the cultures. It has appeared oftenest in fluid cultures about two weeks old.

The varieties of movements are chiefly rotatory, sometimes undulating, and seldom lashing. I have never observed lateral vibration such as is commonly seen in active specimens of *Treponema macrodentium*,<sup>5</sup> *Spirochæta obermeieri*,<sup>6</sup> and *Spirochæta gallinarum*,<sup>7</sup> in pure cultures.

The organism stains reddish violet by Giemsa's solution, faintly red by carbol fuchsin, but not by Gram's method.

*Division*.—Longitudinal division has been observed, but transverse division may also occur.

*Pathogenicity*.—Repeated attempts to produce lesions with pure cultures of this organism in the skin of monkeys (*Macacus rhesus*, *Cercopithecus callitrichus*, *Papio babuan*), and in the testicle or cornea of rabbits resulted negatively.

*Differentiation from Allied Species*.—There is no difficulty in differentiating this organism from *Treponema microdentium*<sup>8</sup> and

<sup>5</sup> Noguchi, *Jour. Exper. Med.*, 1912, xv, 81.

<sup>6</sup> Noguchi, *idem*, 1912, xvi, 199.

<sup>7</sup> Noguchi, *idem*, 1912, xvi, 620.

<sup>8</sup> Noguchi, *idem*, 1912, xv, 81.

*Treponema mucosum*,<sup>9</sup> as both the latter are much smaller and produce an unpleasant odor in culture (figures 10, 11, and 18.) Differentiation from *Treponema pallidum* in culture offers no difficulty when one is dealing with the average and thinner types of the pallidum,<sup>10</sup> because this organism is altogether too thick to be confused with the average specimens of pallidum. On the other hand, a certain precaution is required when one is dealing with a strain of pallidum belonging to the thicker type.<sup>11</sup> In this instance, especially when one lacks means of comparing both species in pure culture under exact and parallel cultural conditions, the two are apt to be confused, for it is not impossible that, by providing one species with one condition and the other with another, the morphological features will approach each other so nearly that considerable resemblance may result. In my cultivation work this difficulty did not arise, because most of my strains of the cultivated pallidum belonged to the average type.<sup>12</sup> Besides, in cases when the heavier pallidum strains were obtained from the testicular lesions of rabbits, there was no possibility of obtaining the above species, and when a dubious strain was isolated directly from human chancres or condylomata, only that which produced characteristic lesions in animals (monkeys) was designated as the pallidum.<sup>13</sup>

Since obtaining two strains of this organism in pure cultures I have compared it with the thicker type of the pallidum under parallel cultural conditions. The study showed that under the same conditions even the thick type of pallidum is still decidedly thinner than the specimens here described. Under unfavorable conditions, especially in fluid medium, the width of the pallidum (thick type) may reach in some specimens that of the thinnest of the spirochætæ in question, but the majority of pallida have a width of about 0.3 of a micron, while in the latter 0.4 to 0.45 is the average. In suitable solid medium, the thick pallidum never exceeds 0.3 of a micron, while the other specimens measure usually 0.35 to 0.4 of a micron, with few exceptions. Moreover, the curves of the pallidum are

<sup>9</sup> Noguchi, *Jour. Exper. Med.*, 1912, xvi, 194.

<sup>10</sup> Noguchi, *idem*, 1912, xv, 201.

<sup>11</sup> Noguchi, *idem*, 1912, xv, 201.

<sup>12</sup> Noguchi, *idem*, 1912, xv, 201.

<sup>13</sup> Noguchi, *idem*, 1912, xv, 90.



more closely set and more rigid than those of the present organism. Another point of difference between the two species is that in ascitic agar the pallidum never grows without fresh sterile tissue, while the other can grow without tissue.<sup>14</sup>

The next species entering into consideration are *Spirochæta balanitidis*<sup>15</sup> and *refringens* and *Treponema macrodentium*. *Spirochæta balanitidis* is described as having a width of 0.5 to 7.5 microns and a length of 10 to 12 microns. Its curves are rather regular and quite deep. Besides, the balanitidis is described as having a band-like body, an undulating membrane, and a periblastic projection. The present species is decidedly thinner, has a cylindrical body, and no undulating membrane. *Spirochæta refringens*<sup>16</sup> is much coarser and has wavy, irregular curves, although in pure cultures the curves may be quite regular in some specimens (figure 9). The appearance of the growth and the cultural conditions required are similar for the refringens and the present species. *Treponema macrodentium*<sup>17</sup> resembles the present organism in length, but differs from it because of slightly less width, tapering body, irregular curves, creeping and lateral vibratory movements (figure 8). When viewed under the dark-field microscope there is no difficulty in distinguishing them. Further, the macrodentium in pure culture never grows without the tissue.

*Identification.*—In the foregoing pages I have distinguished this organism from *Treponemata pallidum*, *microdentium*, *mucosum*, and *macrodentium*, and from *Spirochæta refringens* by means of morphological and biological properties present in pure cultures. It

<sup>14</sup> Whether or not a strain of pallidum which has suffered a marked morphological and biological deviation from the original culture, such as appears to be the case with the strain I sent to Paris, will grow in a medium without tissue, I cannot say. This strain in my possession still remains unmodified since its isolation from a condyloma latum a little over a year ago. The preservation of morphological and biological characteristics in my hands is undoubtedly due to the more strictly parasitic condition provided in my culture medium, while the deviation of the same strain from the original type in the hands of Levaditi and Danulesco (*Compt. rend. Soc. de biol.*, 1912, lxxiii, 256) can easily be explained by the modification of cultural methods and media adopted by these authors.

<sup>15</sup> Hoffmann, E., and von Prowazek, S., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1906, xli, 741.

<sup>16</sup> Noguchi, *Jour. Exper. Med.*, 1912, xv, 466.

<sup>17</sup> Noguchi, *idem*, 1912, xv, 81.

was further differentiated from *Spirochæta balanitidis* from the description and photographs given by Hoffmann and von Prowazek. The question now arises whether or not the present species corresponds with some other organisms described by previous investigators.

Mulzer in 1905 described a small spirochæta resembling the pallidum occurring in cases of ulcerating carcinoma of the uterus or breast and proposed the name of *Spirochæta pseudopallida*.<sup>18</sup> This organism may have been identical with the variety found in cases of carcinoma by Hoffmann,<sup>19</sup> Krienitz,<sup>20</sup> Loewenthal,<sup>21</sup> Kiolemenoglou and von Cube,<sup>22</sup> Scholtz,<sup>23</sup> Loewy,<sup>24</sup> and Schmorl.<sup>25</sup> Krienitz's organism is called *Spirochæta microgyrata*, while that of Hoffmann is called *Spirochæta gangrænæ carcinomatosæ*. The lack of the descriptions regarding their behavior in the living state and of other biological properties renders it impossible to identify the present organism with any of these. In 1907 Veszprémi<sup>26</sup> proposed the name *Spirochæta gracilis* for a small organism found in an ulcerating neoplasm of the jaw which communicated with the buccal cavity. The description of the morphology and the figures of the organism make it probable that the organism described by him belongs to the dentium varieties and is especially allied to *Treponema macrodentium*. At all events, *Spirochæta gracilis* cannot be identified with the present organism. In 1909 Levaditi and Stanesco<sup>27</sup> obtained in mixed cultures a spirochæta from syphilitic as well as non-syphilitic lesions and pus occurring on the human genitalia.

<sup>18</sup> Mulzer, P., *Berl. klin. Wchnschr.*, 1905, xlii, 1144.

<sup>19</sup> Hoffmann, E., *Berl. klin. Wchnschr.*, 1905, xlii, 880; *Deutsch. med. Wchnschr.*, 1906, xxxii, 967; *Atlas der ätiologischen und experimentellen Syphilisforschung*, Berlin, 1908.

<sup>20</sup> Krienitz, W., *Centralbl. f. Bakteriöl., 1te Abt., Orig.*, 1906, xlii, 43.

<sup>21</sup> Loewenthal, W., *Berl. klin. Wchnschr.*, 1906, xliii, 283.

<sup>22</sup> Kiolemenoglou, B., and von Cube, F., *München. med. Wchnschr.*, 1905, lii, 1275.

<sup>23</sup> Scholtz, W., *Deutsch. med. Wchnschr.*, 1905, xxxi, 1487; 1910, xxxvi, 215.

<sup>24</sup> Loewy, K., *Arch. f. Dermat. u. Syph.*, 1906, lxxxi, 107.

<sup>25</sup> Schmorl, G., *München. med. Wchnschr.*, 1907, liv, 188; *Deutsch. med. Wchnschr.*, 1907, xxxiii, 876.

<sup>26</sup> Veszprémi, D., *Centralbl. f. Bakteriöl., 1te Abt., Orig.*, 1907, xliv, 332, 408, 515, 648.

<sup>27</sup> Levaditi, C., and Stanesco, V., *Compt. rend. Soc. de biol.*, 1909, lxvii, 188.

This organism was described as having a width of 0.33 to 0.5 of a micron, a length of 3.5 to 15.5 microns, as presenting fairly regular curves, and as being more actively motile than the pallidum and staining bluish red with Giemsa's solution. As regards pathogenicity no conclusion could be drawn, as they used mixed cultures to produce an acute inflammation of the prepuce of a chimpanzee, and did not rule out the part played by contaminating bacteria in the process. They ascribe the inflammatory process to their spirochæta, which they call *Spirochæta gracilis*. They did not identify their organism with Veszprémi's, previously so named, or make any reference to his previous work. Thus if Levaditi and Stanesco applied this name to their organism without knowing that it was already used by Veszprémi, and if their organism was not identical with that described by this author, they must seek a new appellation. On the other hand, if they had identified their organism with that of Veszprémi, the species we are describing is clearly different from theirs. Judging from the description and also the source from which Levaditi and Stanesco obtained their mixed cultures, I am, however, inclined to think that their organism was probably identical with that described by me in the present article.<sup>28</sup> Even if their organism were the same as mine, it would be impossible to accept the name *Spirochæta gracilis* for this species as, in doing so, it would be erroneously identified with that of Veszprémi. On this account, and since I have obtained the new species in pure culture and studied its properties systematically, I feel justified in proposing as its name *Treponema calligyrum*.<sup>29</sup>

#### CONCLUSIONS.

1. On the surface of genital or anal lesions, either syphilitic or non-syphilitic, there may be found occasionally a spirochæta resem-

<sup>28</sup> E. Hoffmann, in his latest publication (Finger, E., Handbuch der Geschlechtskrankheiten, Vienna, 1912, 828), remarks that while *Spirochæta balanitidis* and *Spirochæta refringens* are much coarser organisms than *Treponema pallidum*, yet there may be found some specimens which are quite thin and may cause difficulty in being distinguished from the pallidum. He does not state, however, whether the thin forms are to be considered as an independent variety or only atypical forms of the refringens or balanitidis.

<sup>29</sup> I take pleasure in acknowledging my indebtedness to Professor Gonzalez Lodge of Columbia University for selecting a suitable adjective.

bling *Treponema pallidum*, but somewhat thicker than the latter. In general characteristics, this organism occupies an intermediary position between *Treponema pallidum* and *Spirochæta refringens*.

2. The treponema was obtained in pure culture from two cases. For the organism the name of *Treponema calligyrum* is proposed.

3. *Treponema calligyrum* is non-pathogenic for monkeys and rabbits.

4. *Treponema calligyrum* can be distinguished from *Treponema pallidum*, *Treponema microdentium*, *Treponema macrodentium*, *Treponema mucosum*, and *Spirochæta refringens* either by the morphology, cultural and biological properties, or all these conditions in combination.

5. Under certain cultural conditions *Treponema calligyrum* becomes thinner and may cause some difficulty of differentiation from a strain of the thick type of *Treponema pallidum*, especially when the latter is grown under conditions which tend to render it thicker. There is no such difficulty when the average and thin types of *Treponema pallidum* are concerned. On the other hand, by providing the calligyrum with conditions which lead to the appearance of thicker specimens and by supplying *Spirochæta refringens* with conditions of growth that favor the development of individuals that are thinner and more regularly curved, the refringens may be made to resemble the calligyrum in general appearance; but under identical cultural conditions they can be readily distinguished.

#### EXPLANATION OF PLATES.

##### PLATE 6.

FIGS. 1 and 2. *Treponema calligyrum* in pure culture, ten days old, in solid medium. Giemsa stain.  $\times 1,100$ .

FIGS. 3 and 4. *Treponema pallidum* in pure culture, four weeks old, in fluid medium. Giemsa stain.  $\times 1,100$ .

FIG. 5. *Treponema pallidum* from orchitis of the rabbit. Giemsa stain.  $\times 1,100$ .

##### PLATE 7.

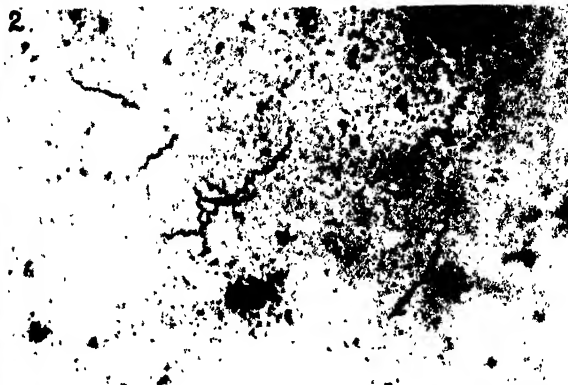
FIGS. 6 to 11. Schematic reproduction of dark-field views of various species of *Treponema* in pure culture. FIG. 6. *Treponema calligyrum*; FIG. 7. *Treponema pallidum*; FIG. 8. *Treponema macrodentium*; FIG. 9. *Spirochæta refringens*; FIG. 10. *Treponema microdentium*; FIG. 11. *Treponema mucosum*.

FIGS. 12 to 14. *Treponema calligyrum* in pure culture, ten days old, in solid medium. Dark-field view.  $\times 1,100$ .

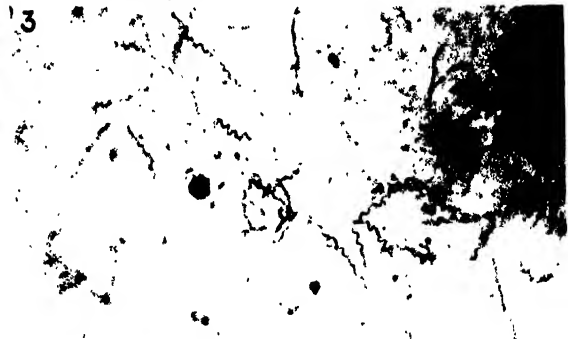
FIGS. 15 to 17. *Treponema pallidum* in pure culture, four weeks old, showing morphological variations among different strains. Dark-field view.  $\times 1,100$ .

FIG. 18. *Treponema microdentium* in pure culture, ten days old, in solid medium. Dark-field view.  $\times 1,100$ .

Calligyrum - Pure Culture



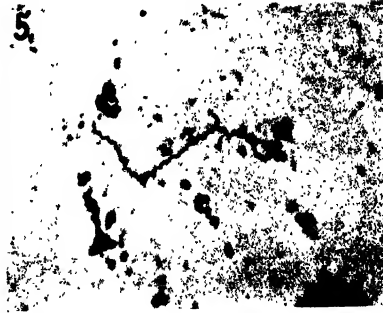
Pallidum - Pure Culture



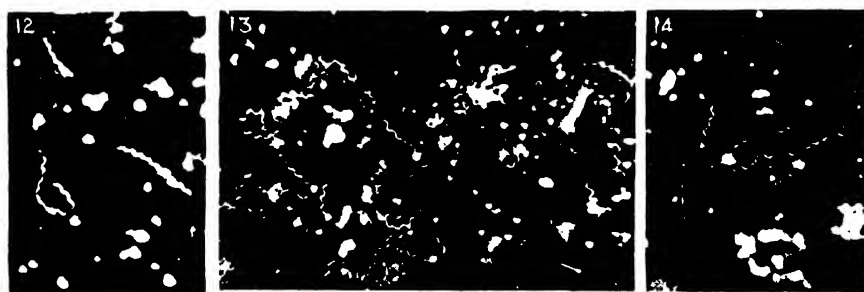
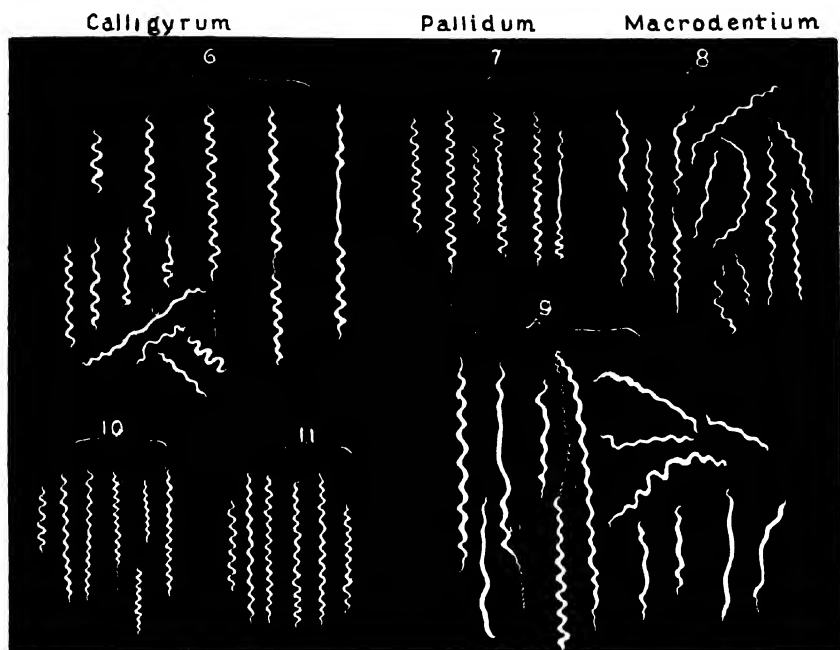
Pallidum-Pure Culture



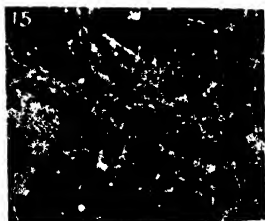
Pallidum in Tissue







Calligyrum



Pallidum

Microdentium





## EPIDEMIC POLIOMYELITIS.

### FOURTEENTH NOTE: PASSIVE HUMAN CARRIAGE OF THE VIRUS OF POLIOMYELITIS.

By SIMON FLEXNER, M.D., PAUL F. CLARK, Ph.D., AND  
FRANCIS R. FRASER, M.B.

*(From the Laboratories and Hospital of The Rockefeller Institute for Medical Research, New York.)*

The manner of conveyance of the virus of poliomyelitis to infected persons is not yet wholly worked out. The presence of the virus has been established on the mucous membrane of the nose, throat and intestines of persons ill of poliomyelitis, and on the mucous membrane of the nose and throat of monkeys in which the disease has been produced experimentally. Aside from infected persons and monkeys the virus has once been found in external nature; namely, in sweepings obtained from a room in which patients with poliomyelitis had recently been kept (Neustaedter and Thro). The latter finding indicates that the virus is capable of leaving the infected body in a manner that permits of its persistence as dust. Since the virus has been shown to occur frequently, if not constantly, in the discharges of the nose and throat, it is clear that it must escape into the surroundings of the infected, where, when dried, it would retain its activity for a considerable period of time.

Clinical observation has awarded a definite rôle to healthy human carriers of the poliomyelitic infection, but thus far indubitable experimental demonstration of the occurrence of such passive carriers has not been brought. The obstacles in the way of the demonstration are considerable but not insuperable. Since the virus may leave the infected body by the nasal and buccal discharges, it is to be presumed that if carried and transmitted at all by healthy persons, it would be through the medium of these discharges. This definition does not exclude the soiled hands, objects, etc., from being also

a means of transportation. The investigation of the discharges mentioned offers the readiest opportunity to determine the presence of the virus. We possess at present one means only of detecting the virus, and that is its implantation on monkeys, in which it is capable of producing an experimental form of poliomyelitis resembling in all essential respects the spontaneous disease, so-called, occurring in man.

Considerable difficulties, however, surround the initial implantation of the virus on monkeys. Even when the human spinal cord, derived from a recent fatal case of poliomyelitis in man, is employed for inoculation, the experimental infection has not always been produced. We now know that the initial infectivity for monkeys of human strains of the virus varies greatly. In order to secure infection with this human material, large quantities of an emulsion may be required for inoculation. When such emulsions, sufficient to cause the experimental disease, are filtered through a Berkefeld or other candle, the resulting filtrate may fail to cause infection. Moreover, in the successful transfer of the disease from originally infected monkeys to other monkeys, certain strains of the virus have proved more readily inoculable than others.

It is to be presumed that passive carriers will harbor the virus in smaller amount than persons actively ill of poliomyelitis. In dealing with the discharges from the nose and throat it is practicable to employ for inoculation only washings that have been made bacteria-free by filtration. The filtrates consist of saline washings of the upper respiratory mucous membrane and therefore represent at best highly dilute suspensions of the human race of the virus, so to speak. The inoculations are best made simultaneously into the sheath of the sciatic nerves and the peritoneal cavity, or into the brain and peritoneal cavity, and the quantity of fluid employed for the injections may be as great as 200 c.c.

Monkeys possess a certain grade of resistance to infection with the virus of poliomyelitis, even when it is introduced directly into the brain or the nerves. This resistance is readily overcome by a highly active strain of the virus, the most active strains being those which have been passed successively through a large number of monkeys. It is overcome with difficulty, if at all, by the least active

human strains. Hence the probability of highly dilute filtrates of human strains of virus causing infection in the monkey is not great.

Besides the frank and unmistakable instances of flaccid paralysis, constituting the most impressive symptom of poliomyelitis, a less definite set of symptoms has been described indicating the abortive form of the disease. It has happened in our experience that such weak filtrates as have been described have failed to produce frank paralysis and yet have given rise to the more indefinite set of symptoms. These relatively slight and indefinite forms of illness following the inoculation do not permit of a conclusion as to the nature of the infection. To prove by immunity tests that they have been instances of true poliomyelitis of a mild form is difficult and not convincing. Nothing less than frank, typical instances of the disease, associated with characteristic lesions in the spinal cord, etc., should be accepted, under the circumstances, as evidence of successful infection. Moreover, this preliminary successful result should be confirmable through reimplantation of the virus, now contained in the spinal cord of the paralyzed monkey, on still other monkeys, in which frank paralysis should occur, and in which the central nervous system should exhibit the characteristic effects of the disease. When these tests have been successfully carried out, we may conclude the demonstration of the presence of the virus to be complete.<sup>1</sup>

1. It is necessary here to refer to the large and carefully conducted series of tests of suspected human carriers of the poliomyelitic virus made by Kling, Pettersson and Wernstedt (Report of the State Medical Institute of Sweden to the Fifteenth International Congress on Hygiene and Demography, Washington, 1912) in 1911. These investigators believe that they have established the important fact that such carriers occur and are several times more numerous than the frank and abortive cases of poliomyelitis combined. An obstacle to the full acceptance of the conclusions reached by Kling, Pettersson and Wernstedt exists, and that is the absence from the inoculated monkeys, many of which seem to have shown obvious clinical symptoms of poliomyelitis, of characteristic alterations of the central nervous system. These investigators describe a new set of lesions of the spinal cord and medulla, affecting<sup>o</sup> chiefly the glia and nerve-cells and consisting of hypertrophy of the former and hyaline and vacuolar degeneration of the latter, which may even be encroached on by glia cells. Similar lesions have not been observed in fatal cases of poliomyelitis in man, and their specific relation to poliomyelitis should be regarded as still an open question. Account should be taken also of the fact that the monkey organs showing these lesions were reinoculable only occasionally and did not cause, in the second

In view of the facts related, it is patent that in dealing with washings containing discharges from the upper respiratory tract of persons in contact with active cases of poliomyelitis, failure is much more likely to result than is success. For the same reason we must conclude that failure to obtain infection in the monkey cannot be accepted as positive evidence of an absence of the virus from the human materials examined. These considerations, on the other hand, give to a successful result a somewhat intensified value. When it is achieved, it may be taken not only as establishing the occurrence of the virus, but also as indicating its presence in relatively large amount, or the possession of a higher degree of virulence for monkeys than is ordinarily displayed.

We wish to describe an instance of the demonstration of the virus of poliomyelitis on the upper respiratory mucous membranes of healthy human adults, the parents of a child suffering from an acute attack of epidemic poliomyelitis.

E. A., a girl aged 4 years and 4 months, was somewhat ill from October 12 to 17, 1912, when fever and weakness were especially noted. During the evening of the 17th partial paralysis of the legs developed. She was admitted to the hospital of the Rockefeller Institute October 18, at which time the left leg could not be drawn up and was flaccid. Some power remained in the hamstring muscles. The right leg was less affected; it could be drawn up weakly and again extended. Below the knee, however, the muscles were all weak. The respiratory muscles were definitely affected, the right more than the left. The movement of the chest during respiration was almost imperceptible. On account of extraneous causes the child was discharged October 28, at which time improvement had already begun in the intercostal muscles and those of the right leg.

The mother and father of E. A. were subjected to a nasopharyngeal irrigation with normal saline solution October 28. About 150 c.c. of fluid were collected, shaken, and pressed through a Berkefeld filter. This fluid was introduced into a *Macacus cynomolgus* as follows: about 1.5 c.c. were injected into the sheaths of each sciatic nerve, and 140 c.c. into the peritoneal cavity. Recovery from the anesthetic was prompt, and the animal remained well until November 11, when it was noted to be excitable and to drag the right leg. The left leg proved to be weak. November 12 the right leg was definitely flaccid. A lumbar puncture yielded 2.5 c.c. of fluid containing an excess of white corpuscles. November 13, condition unchanged; etherized. The autopsy showed no

generation in monkeys, the development of characteristic histologic effects. These authors ascribe the atypical lesions to a poliomyelitic virus of diminished virulence. This assumption is probably capable of being confirmed or set aside by successive inoculations of the infected nervous tissue and for the purpose of enhancing its virulence.

lesions at the inoculation sites, but the spinal cord appeared edematous. The general viscera were normal in appearance. Portions of the spinal cord and medulla were taken for sections and for preservation in sterile 50 per cent. glycerin solution. The sections showed typical lesions of experimental poliomyelitis, consisting of perivascular, mononuclear infiltration, infiltration of the ground substance of the gray matter, and necrosis and phagocytosis of the ganglion cells of the spinal cord and medulla. December 3 an emulsion of the glycerinated spinal cord of this monkey was injected into each sciatic nerve and into the peritoneal cavity of a *Macacus rhesus* and a *Macacus cynomolgus*. Recovery from the anesthetic was prompt and the animals remained well until December 9, when the rhesus was noted to be excitable. On the 10th a lumbar puncture yielded 3 c.c. of slightly opalescent fluid under increased pressure. It contained an excess of white corpuscles; the globulin reaction was negative. On the 13th the excitability was still present and the legs were moved awkwardly. On the 17th the excitability had diminished and a lumbar puncture yielded 2 c.c. of opalescent fluid giving a faint reaction for globulin with Noguchi's butyric acid test, and showing an excess of white corpuscles. The animal was etherized. Sections of the spinal cord showed infiltrative lesions of poliomyelitis. The cynomolgus became excitable December 10, the condition persisting until the 16th when it was diminished. On the 19th the right leg was partly paralyzed and the left leg was weak. Lumbar puncture gave fluid containing an excess of white corpuscles. On the 20th the right leg was completely flaccid; excitability had increased, and a general tremor was present. On the 21st the right leg was still weaker, and the left leg, right leg, right arm and back were weaker. On the 23d both arms were weak; the animal lay down much of the time. It was etherized. The autopsy showed edema and congestion of the spinal cord, while the organs generally appeared normal. Sections of the cord, medulla and intervertebral ganglia contained typical infiltrative and ganglionic cell lesions, characteristic of poliomyelitis.

The foregoing protocols leave no doubt that the washings from the nasopharynx of the parents of E. A., neither of whom showed any symptoms of illness and who were evidently not suffering from poliomyelitis, contained the virus of epidemic poliomyelitis. The result described brings indubitable evidence of the occurrence of the virus of the disease in the nasopharynx of healthy persons who have been in close contact with an acute case of poliomyelitis, and affords an experimental basis for the belief, based on clinical observation, of the occurrence of passive human carriers of the infection.

## EXPERIMENTS ON THE CULTIVATION OF THE VIRUS OF POLIOMYELITIS.

### FIFTEENTH NOTE.

BY SIMON FLEXNER, M.D., AND HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York.*)

It has not hitherto been found possible to cultivate the virus, or etiologic cause, of epidemic poliomyelitis outside the animal body. Flexner and Lewis<sup>1</sup> noted that when a Berkefeld filtrate, prepared from the infected nervous system of the monkey, was mixed with ascitic fluid and the mixture incubated a turbidity arose; but they could not determine that the turbidity was caused by multiplication of a living microorganism. Only the original mixture, and not the subcultures, was capable of infecting monkeys.

Recently the efforts at cultivation of the virus have been resumed. Encouraged by the cultivation of the spirochetes by Noguchi we concluded to apply the methods worked out by him<sup>2</sup> to the investigation of poliomyelitis. Since the results achieved so far are of quite a different order from those obtained previously, we shall describe them briefly here.

The virus belongs to the class of filterable organisms and passes readily through Berkefeld filters (V and N). Up to the present it has not been rendered certainly visible, although we now know that "filterable" and "ultramicroscopic" are not equivalent terms.

We have employed for our experiments tissues from the brain and spinal cord of human beings who have died of epidemic poliomyelitis, and of monkeys the subjects of the experimental disease. Part of the tissues had been preserved for many months in 50 per cent. glycerin solution and were free from ordinary bacterial contamination, as were the fresh tissues.

1. Flexner, Simon, and Lewis, Paul A.: Epidemic Poliomyelitis in Monkeys, THE JOURNAL A. M. A., Jan. 1, 1910, p. 45.

2. Noguchi, Hideyo: Jour. Exper. Med., 1911, xiv, 99; 1912, xv, 90; 1912, xvi, 109, 211.

The cultivations have been conducted both with Berkefeld filtrates and tissues in substance. The culture mediums consist, first, of sterile, unfiltered ascitic fluid or of brain extract to which fragments of sterile rabbit kidney and a layer of paraffin oil have been added, and of these plus 2 per cent. nutrient agar-agar in proportions of 1 to 2. The first mediums permit of a slow growth not visible to the naked eye, while the second (which are unsuitable for obtaining the initial growth) yield, after several days, visible minute colonies clouding the tubes. The cultivations are conducted under anaerobic conditions, and the colonies do not ascend to the summit of the deep layer of solid medium.

The minute colonies are composed of globular or globoid bodies, averaging in young cultures 0.15 to 0.3 micron in size. The bodies appear in a variety of arrangements: single, double, short chains and masses. Often they appear embedded in a material of different refractive index. In older cultures certain bizarre forms have been noted. The cultivated bodies stain a pale reddish violet in Giemsa's solution, and bodies of identical appearance have been demonstrated by Noguchi, also with Giemsa's solution, by a specially devised method in films prepared directly from the nervous tissues.

Monkeys have been inoculated with the cultures. Two series of inoculations have been made and a third series is in progress. Cultures from human tissues in the third, and from monkey tissues in the fifth generation have caused typical experimental poliomyelitis in the monkey. The findings at autopsy and the microscopic appearances of sections of the spinal cord, medulla and intervertebral ganglia were characteristic of the disease. From the nervous tissues of these animals other monkeys were successfully inoculated and pure cultures recovered.

The third series of experiments in progress consists of inoculations of later generations of the cultures, the object of which is to exclude the carrying over, with the cultures of the globoid bodies, of a quantity of the original virus sufficient to cause paralysis in the inoculated monkeys. This effect of the accidental carriage of the virus is more likely to arise in connection with the highly virulent "monkey" strain than with the relatively weak human strain, although it is perhaps not wholly impossible even with the latter.



## VARIATIONS IN A CHICKEN SARCOMA CAUSED BY A FILTERABLE AGENT.\*

By PEYTON ROUS, M.D., AND JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

### PLATES 41 TO 49.

Marked alterations in structure and behavior are not uncommon in tumors which run their course in a single individual; but, as might be expected, they are more frequent and better marked in growths of which the existence is prolonged by transplantation. The present paper has to do with the changes that have taken place in a spindle-celled sarcoma of the fowl<sup>1</sup> during its propagation for a period of about three years, and in numerous individuals. The findings have especial claim to attention because the sarcoma, otherwise a typical tumor, can be transferred to new hosts, not only by transplantation, but by means of an agent which is separable from the neoplastic cells, and is probably a living organism. Possibilities of variation are thus introduced which do not appear in the case of tumors that depend for their transmission solely upon the survival of a parent strain of cells.

In several previous communications, the characters of the chicken sarcoma have been taken up in detail.<sup>2</sup> Under ordinary circumstances its growth takes place by a proliferation of the cells already neoplastic, and apparently in that way only; while the metastases are referable entirely to autotransplantation. The transmission of the disease to other susceptible hosts is most readily accomplished by implanting in them a bit of the sarcomatous tissue, which, as experiment has shown, will survive in the new site, and by its proliferation give rise to the new tumor. The growth is at present developing in

\* Received for publication, November 13, 1912.

<sup>1</sup> Rous, P., *Jour. Exper. Med.*, 1910, xii, 696; 1911, xiii, 397.

<sup>2</sup> Rous, P., *loc. cit.*; Rous, P., and Murphy, J. B., *Jour. Am. Med. Assn.*, 1912, lviii, 1938; *Jour. Exper. Med.*, 1912, xv, 270; Rous, P., Murphy, J. B., and Tytler, W. H., *Jour. Am. Med. Assn.*, 1912, lviii, 1751, 1840.

the thirty-second successive series of fowls to which it has been thus transferred. Despite our knowledge that the individual neoplasms arise practically *in toto* through a proliferation of the cells introduced from the last host, it is uncertain whether the present tumors are strictly the offspring by cell division of the original growth. For a transforming action of the causative agent upon normal tissue which would be negligible in the individual case might well bring about in the course of time and many transplantations a total change in the growth's make-up. At all events the tumors of the thirty-two series of transplantations represent the most direct possible continuance of the original sarcoma strain. In addition, many growths have been studied which were produced by the causative agent acting alone, either as a filtrate or as dried or glycerinated tumor tissue, and many more which may have resulted either from an action of the agent or the survival of tumor cells, since they developed after the implantation of sarcomatous tissue damaged *in vitro* but not killed. In all, the primary and secondary growths of 217 fowls have been carefully gone over.

#### LOCAL VARIATIONS.

The structural variations occurring under ordinary conditions in different parts of the same sarcoma will first be taken up. Certain of these, due to degeneration or to attempts at differentiation, are found in nearly every specimen.

A typical growth consists of spindle cells, more or less attenuated, coursing this way and that in irregular strands. When rapid proliferation is taking place the cells near the border of the growth may be short and blunt, or even irregularly rounded (figure 1). As the mass increases in size and the cells once at its edge come to lie deep within it, they cease to divide so actively, gradually assume an attenuated spindle form, and produce intercellular fibrils. A similar progressive differentiation is often noted in the tumors of human beings.

For the most part the active cells in the best nourished portions of the sarcoma are the ones which enter the blood vessels and are distributed to distant parts (figure 2). The secondary foci partake of the character of the emboli from which they arise, and they may

consist, when small, entirely of active cells of blunt or rounded form (figure 3), appearing for this reason markedly different from the older tissue of the primary mass. But as time passes and the metastases enlarge, their cells gradually become spindle-shaped like those in the parent growth. In many instances the metastases consist from the beginning of spindle cells.

Degenerative changes are of several sorts. In old tumors the spindle cells may become extremely attenuated (figure 4), if necrosis does not overtake them. More frequently cell death occurs early, often preceded by a hydropic change. In the latter case the cells may become greatly swollen and multinucleated. A myxomatous change is not infrequent, and is seen especially at the center of fairly nourished, slowly growing tumors in resistant hosts. It cannot be looked upon as intermediate to the ordinary necrosis.

Very striking accommodative changes are sometimes seen. Their dependence on special conditions of pressure and nutrition in the organ containing the tumor is usually very evident. In cross section growths in voluntary muscle have often an alveolar structure,<sup>8</sup> the result of replacement of the muscle fibers with retention of their outline. Growths in the lungs may present a very complex picture. The pulmonary air spaces may contain masses of actively dividing spheroidal tumor cells, easily distinguishable from desquamated epithelium; whereas nearby in the interstitial tissue the neoplastic cells are of spindle form in strands (figure 5). The structure of the ovary leads to a somewhat similar diversity there. In the liver, kidney, spleen, and heart the sarcoma is evenly constituted of spindle cells. In the gizzard, an organ of very close texture, the growth is often non-invasive, and its spindle cells are compactly arranged and often are attenuated. All these various pictures may be seen in the metastases of a single tumor. It is not improbable that pressure changes have something to do with the gradual development of the attenuated spindle form in the older portions of primary growths.

Certain striking histological differences are sometimes observed in secondary tumors seated close to one another in the spleen or liver. Of two neighboring foci in the liver one may consist of sharply defined, rounded, or even cuboidal cells, arranged in columns within

<sup>8</sup>Rous, P., *Jour. Exper. Med.*, 1911, xiii, 397.

the portal spaces, and superficially very suggestive of newly formed bile ducts; while the other has the usual spindle-celled form. In the spleen there are sometimes found, in addition to nodules of the ordinary sarcoma, sharply circumscribed growths consisting entirely of short, closely crowded, fusiform cells (figure 6) in which mitoses are unusually numerous.

At first sight it would seem unlikely that these diverse secondary nodules are true metastases of a single tumor. Instead it might be assumed that they have arisen in part by localizations of the sarcoma-producing agent in connective tissue cells of unusual potentiality, lying within the affected viscus. But against this assumption are our experiments which show that the agent rarely if ever acts to cause secondary nodules,<sup>4</sup> and the direct proof that these latter are in general the result of a dissemination of cells from the primary growth. Furthermore all morphological gradations can be traced between the ordinary sarcoma and the peculiar tumors, and as the latter enlarge, their cells take the spindle form. When one considers how markedly local differences in nutrition and pressure influence the appearance of the sarcoma, and the fact that the histology of each beginning metastasis depends to some extent on the character of the embolus from which it has arisen (embolus of rounded cells, embolus of spindle cells), the assumption of a secondary localization of the agent seems unnecessary. On the other hand, the occasional occurrence of such a localization can not be absolutely ruled out.

#### CHANGES WITH INCREASED MALIGNANCY.

Some of the most marked changes in the sarcoma have accompanied its increase in malignancy. The original tumor was a discrete, fairly encapsulated mass, firm and gristly, with a striated cut surface. At its center was an irregular core of coagulation necrosis. The tumors of the first few generations were quite similar, but often contained one or more cysts full of pale yellow, mucinous fluid. With the great increase in transplantability and rapidity of growth which took place between the second and sixth tumor gener-

<sup>4</sup>Rous, P., and Murphy, J. B., *Jour. Exper. Med.*, *loc. cit.*; Rous, P., Murphy, J. B., and Tytler, W. H., *loc. cit.*

ations, the character of the neoplastic tissue altered markedly. It became soft, friable, and translucent, with little striation and no encapsulation. Rapid breaking down *en masse* occurred often, and in these cases amyloid was sometimes found in the spleen and liver.<sup>5</sup> Hemorrhage into the tumor substance with the formation of large, ragged cavities full of brown or green or bloody fluid was very frequent. The cells had often a very irregular spindle form, or were short and blunt or even rounded. Their increased activity is perhaps best illustrated by the change in their behavior toward striped muscle. In the earlier generations they had supplanted this by penetrating in strands between its fibers, bringing about their gradual atrophy and disappearance; but now they often apposed themselves directly to the fiber substance, which disappeared before them as though eroded; or else they penetrated the sarcolemma at one or more points and proliferated and extended rapidly within it<sup>6</sup> in a manner met with in only the most malignant of mammalian growths.

The gross form of the neoplasm was much altered with the increase in malignancy, as would naturally follow from its greater invasive power. The earlier series of growths resulting from implantation in the pectoral muscles were discrete, and frequently projected sharply from the body contour. But the later ones at this site have been much less definite in form, though still considerably firmer than the normal tissue and easily distinguished on palpation. They may first become evident to the naked eye as diffuse swellings which little by little gain in prominence through expansive growth and the emaciation of the host. Only in somewhat resistant fowls is the very discrete character of the original attained. In these the sarcoma may be encapsulated and very firm, owing to the large amount of non-neoplastic, fibrous tissue about and within it. No better example of tumor malignancy as affecting tumor form could be desired. The course of a growth in the individual fowl may be predicted with considerable accuracy from the findings on a single palpation.

<sup>5</sup> For amyloid changes in the organs of mice with tumors, see Lubarsch, O., *Centralbl. f. allg. Path. u. path. Anat.*, 1910, xxi, 97.

<sup>6</sup> Rous, P., *Jour. Exper. Med.*, 1911, xiii, 397.

## INTERCURRENT VARIATIONS.

By selective transplantation the sarcoma has been kept for many months in a highly malignant state. From time to time without evident cause changes in its morphology have manifested themselves. In some hosts the tumor consists of spindle cells much smaller than in other fowls of the same age and variety; and in some the growth closely approaches in appearance a round-celled sarcoma (figure 7) though many of its cells have protoplasmic tails or queues, and there is always some differentiation to the spindle form. Most striking are the giant-celled growths.

Giant cells were numerous in some portions of the original tumor, as appears from sections of it recently made (figure 8). Nevertheless, in the nodules resulting from transplantations into the original host they were so few that the impression given was that of a practically pure spindle-celled sarcoma. In the subsequent tumors in other hosts, they were for a long time met with only infrequently and these later growths may well be called pure, spindle-celled sarcomata. But in the nineteenth transplantation generation, giant cells again appeared as an important constituent of the tumor and growths characterized by large numbers of them have since been frequent.

In appearance the giant cells (figure 9) are entirely different from those occurring about foreign bodies in the fowl or associated with avian tuberculosis. They are not arranged in foci but lie scattered among the neoplastic spindle cells, from which their development by a process of enlargement, accompanied sooner or later by degeneration, can be directly traced. They may reach a diameter of 100 microns or more, and are usually oval, with one or two blunt processes, or forks, and a single large nucleus, features which give them a superficial resemblance to ganglion cells. At first the cytoplasm is finely granular and faintly basic, as is that of the spindle cells round about; but later, as the cell enlarges, the cytoplasm stains with eosin and may become vacuolated. The single nucleus, eccentrically placed, is sometimes pyknotic, but more often vesicular and swollen, with its chromatin in a central mass (fish eye nucleus). In the smaller cells mitosis may take place, and in the larger ones

nuclear budding is frequent. Specimens are not rare with from two to forty scattered nuclei of remarkably various size and appearance. Phagocytic activity of the giant cells is frequent. Erythrocytes, leucocytes, or even small tumor cells may be ingested by them. They may be abundant in the best nourished portions of the sarcoma, indeed at its advancing edge (figure 10). They are present, as a rule, in the metastases from primary growths containing them.

The significance of the giant elements is no clearer than in the case of the sarcomatous giant cells of mammals and the conditions which lead to their appearance have not yet been recognized. In stained preparations or under the dark-field microscope they show no distinctive inclusions. During a long period they were observed only in the sarcomata which resulted from transplantation, as distinguished from those engendered by the filterable agent; but of late some change must have taken place in the agent, since now growths caused by it not infrequently contain giant cells. That peculiarities of the host have a considerable determining influence is shown by the fact that a single lot of causative virus, in the form of a filtrate or of dried or glycerinated tumor tissue, will give rise in some fowls to a pure spindle-celled growth, in others to the giant-celled form. In general the giant-celled form is relatively slowly growing, yet in hosts with an evident, though partial, resistance, tumors of either sort may be found. All things considered it would seem probable that the giant cells represent a perversion which may take place in any of the neoplastic spindle cells when under the influence of special conditions. Some of these conditions are supplied by the host, some by the growth's causative element, and others, doubtless, by local circumstances. The cells belong to the second group of giant cells in sarcomata distinguished by Mallory;<sup>7</sup> namely, to the group of giant cells arising out of tumor cells, in contradistinction to those developing in the non-neoplastic stroma.

#### HEMORRHAGIC FORM OF THE SARCOMA.

Hemorrhage in association with the sarcoma first occurred in the eighth tumor generation, some fifteen months from the time of the original transplantations. The growth had attained its max-

<sup>7</sup> Mallory, F. B., *Jour. Am. Med. Assn.*, 1910, IV, 1621.

imum malignancy in the sixth generation. The first bleedings that were noted took place into areas of necrosis at the center of tumor nodules, and were of slight extent and sharply localized. In the ninth and tenth generations several fowls died suddenly of hemorrhage from visceral nodules and this cause of death has since been frequent. The findings at autopsy are remarkable.

The host is nearly always one in which the tumor has grown rapidly and disseminated widely, though, because of the premature termination of the disease, the secondary growths may have had little chance to develop, and emaciation may be but slight. The lung and heart tumors are seldom hemorrhagic. Bleeding in the occasional metastases in the spleen is kept within bounds by the stout capsule of that organ. Extensive hemorrhage in the primary tumor may result in much breaking down there; but the fatal bleeding usually takes place from a liver metastasis, rarely from a focus in ovary or kidney. At autopsy the peritoneal cavity is found to be more or less distended with blood clot, arranged often in layers of different ages and degrees of firmness; and when this clot is lifted it is discovered to have a direct connection with one or several ruptured blebs or hemorrhagic tumor masses on the surface of one of the organs mentioned (figure 10).

The liver substance may be riddled with discrete, spherical cavities up to one centimeter in diameter, filled with fresh or old blood clots, which, reaching the surface, take the form of blebs. That the tumor cells have in some way caused the hemorrhage is usually evident. In one liver all gradations may be found from microscopic foci of the sarcoma in which hemorrhage has just begun, to large blood cysts which show tumor cells in only a relatively small area of their wall, or to large tumor masses which show relatively small hemorrhages in their interior. Not a few of the blood cysts have a zone of pressure necrosis about them without demonstrable tumor; but the absence of neoplastic tissue under these circumstances is scarcely surprising.

When hemorrhage takes place into the sarcomatous ovary it often converts a number of the follicles into large blood cysts, through the rupture of which death may come about. The hemorrhages in the kidney are usually small and well circumscribed, but they may



dissect into the surrounding tissue and be fatal. The intravenous injection into a normal fowl of the tumor-producing agent in the form of a Berkefeld filtrate has led in one instance to the development of a small sarcoma in the kidney, with a dissecting hemorrhage from which the fowl died.

Although the relationship between tumor and hemorrhage can scarcely be questioned in these cases, there are instances in which it is not so evident. Multiple hemorrhages in the liver of the chicken may undoubtedly be due to other causes than the sarcoma. Occasionally we have found them in the fatty liver of fowls which had not been inoculated with the sarcoma but had died of some other disease. In inoculated hosts dying of a large pectoral sarcoma but having visible metastases only in the lungs they are fairly frequent. In these instances, microscopic search usually brings out the presence of minute patches of tumor in association with some at least of the hepatic hemorrhages (figure 11), and there seems little doubt but that all should be attributed to sarcomatous foci, destroyed for the most part by the bleeding they have induced.

The structure of the growths provoking hemorrhage is often somewhat different from that of the ordinary sarcoma. The cells are short and blunt or nearly spherical, and lie loosely grouped; intercellular fibrils are few, and there is little supporting stroma. These circumstances appear to favor a wide extravasation once the rupture of a vessel has occurred. Vessels are not especially abundant, as they are in the hemorrhagic adenocarcinomata of mice, but the tumor cells are evidently invasive and make their way into many of the blood channels.

#### DISTRIBUTION OF THE METASTASES.

One other alteration in behavior observed during the routine propagation of the growth deserves brief mention, namely, the change in the distribution of the metastases. Elsewhere this subject has already been dealt with to some extent.<sup>8</sup> In the earlier generations of the tumor, the heart was most frequently affected after the lungs. In the later ones the heart has been relatively free from secondary growths and the liver has very often shown them. During the fall

<sup>8</sup> Rous, P., *Jour. Am. Med. Assn.*, 1910, 1v, 1805.

and winter months metastases in the ovary have been rare, but now for two successive years they have been a feature of autopsies performed during the spring and summer (figure 12), or, in other words, during that period when the ovary is functioning actively, when trauma in the organ is of daily occurrence, and when its blood supply is greatly increased. The same factors have not infrequently been observed to determine secondary localizations of tumors of the mouse or man, and they may well be effective in the present case. There is some evidence to show a seasonal localization of the chicken sarcoma in the testicle as well as in the ovary, but our observations on this point have been few, since female birds have in general been used as hosts.

#### SARCOMATA CAUSED BY THE FILTERABLE AGENT.

Taken together, the sarcomata produced by direct injection of the filterable agent do not differ from those resulting from the growth of a bit of the transplanted sarcomatous tissue; and they manifest the same variations. Peculiarities of the individual host are important in determining these. A single lot of agent, in the form of dried, or glycerinated, tissue or a Berkefeld filtrate, will produce in some hosts a pure spindle-celled sarcoma, in others a giant-celled growth, and in yet others a growth composed of fusiform or rounded cells, and perhaps hemorrhagic. All are malignant tumors of the connective tissue, grading into one another histologically.

Attempts to obtain growths of unusual morphology by bringing the agent into contact with special types of cells have as yet been unsuccessful, and so too with attempts to obtain modifications in tumor form by attenuation of the agent *in vitro*. The growths caused by the treated agent take a long time to appear, and frequently retrogress, but otherwise they are not peculiar. The same is true of the sarcomata arising after the implantation of sarcomatous tissue which has been damaged by heat or by saponin. That the tumor cells fail to undergo a structural modification under these influences is scarcely surprising for heating does not alter the structure of transplantable mouse tumors.<sup>9</sup>

<sup>9</sup>Loeb, L., and White, E. P. C., *Centralbl. f. Bakt., its Abt., Orig.*, 1910. lvi, 325.

## DISCUSSION.

The variations manifested by the chicken sarcoma are not more striking than certain ones which have been noted in rat and mouse tumors (Bashford, Lewin) and which are due without doubt to intercurrent changes in a single strain of tumor cells. Lewin, for example, describes an adenocarcinoma which within twelve generations assumed the form of a carcinoma solidum, a carcinoma alveolare, and a cancrioid. The various forms of the chicken tumor are all sarcomata which grade into one another histologically. It would be unnecessary to suppose for them any other cause than intercurrent changes in a single strain of tumor cells were it not that an etiological agent for the tumor has been found,—an agent which accompanies the growth at all its stages, and is capable of causing, under special conditions, a neoplastic change in tissue hitherto normal. The presence of this agent leads one to ask whether the variations in the chicken sarcoma are not due in part at least to changes in it, or perhaps to its action on cells of unusual type or potentiality.

The latter possibility can be almost ruled out. Our many experiments to bring about an action of the agent on a tissue other than those it usually affects have uniformly failed. For example, when the agent, as a Berkefeld filtrate, is injected intravenously into fowls with induced lesions of various tissues the resulting tumor always arises from the spindle cells of connective tissue. On the other hand, there is no doubt but that changes in the causative agent lead to some modifications in the sarcomata of which it is the cause. When the agent is attenuated by heat, it gives rise to tumors which grow slowly and retrogress frequently. And whereas formerly the tumors caused by the agent were all spindle-celled sarcomata, now it not infrequently engenders giant-celled growths. Many of the variations in the transplantation sarcomata, expressing themselves as morphological changes or otherwise, may well be due to modifications in the agent under the play of conditions in successive hosts. The question as to whether the transplantation sarcomata observed during the last three years have consisted entirely of descendants of the original strain of tumor cells or are in part the result of a

neoplastic transformation in successive hosts does not affect this matter.

#### SUMMARY.

Variations are described which have from time to time occurred in the structure and behavior of a transplantable, spindle-celled sarcoma of the fowl, a growth caused, as elsewhere shown, by a filterable agent. Of late the growth has frequently given rise to fatal hemorrhages from its substance. In some of the recent, rapidly growing tumors the cells have tended to be spherical, showing only a very tardy and imperfect differentiation to the spindle form. A giant-celled form of the growth is sometimes met with. Despite their diversity the tumors grade into one another and in the final analysis are all to be considered as spindle-celled sarcomata. Attempts to obtain an action of the etiological agent upon cells other than those it usually affects have failed, as have attempts to bring about changes in the histology of the sarcomata by attenuating the agent.

Some of the lesser morphological variations in the sarcoma are undoubtedly due to local conditions in the host, and of the more important changes some have been associated with an increase in the growth's malignancy. For others the determining conditions have yet to be discovered. On the whole the variations described are not more marked than those occasionally manifested by the transplantable mammalian tumors, and traceable to the changes in a single strain of tumor cells during their propagation in successive hosts. In mammals the ultimate reason for these changes is not known. In the case of the chicken tumor some of them are undoubtedly the expression of changes in the growth's causative agent.

#### EXPLANATION OF PLATES.<sup>10</sup>

##### PLATE 41.

FIG. 1. Marginal portion of a sarcoma which is replacing voluntary muscle. The cells at the edge of the growth (to the left of the picture) are for the most part oval or rounded; those further in are definitely spindle-shaped. The replacement of the muscle fibres has resulted temporarily in an alveolar structure. *m* = muscle fibres in process of disappearance; *r* = rounded tumor cells.

<sup>10</sup> All the microscopic specimens were stained with methylene-blue and eosin

FIG. 2. Growth of the sarcoma into the lumen of a blood vessel, showing the rounded type of tumor cell frequently cast off into the blood stream. The vessel is full of nucleated erythrocytes. *e*=endothelial lining of the vessel; *t*=tumor cells loosened from the main mass; *n*=nucleated red cell lying between the tumor cells.

## PLATE 42.

FIG. 3. Colored photograph of a small metastasis in the lung. It consists of rounded cells similar to those in figure 2. Their staining reaction sharply distinguishes them from the pulmonary tissue.

## PLATE 43.

FIG. 4. Attenuated spindle cells of the older portions of the chicken sarcoma.

## PLATE 44.

FIG. 5. Portion of a diffuse metastasis in the lung. *b.v.*=vessels filled with nucleated erythrocytes; *a.s.*=rounded cells in the air spaces; *s.p.*=spindle-shaped tumor cells in the pulmonary tissue.

## PLATE 45.

FIG. 6. Peculiar type of tumor tissue occurring in some metastases of the spleen.

FIG. 7. Portion of a sarcoma the cells of which are nearly spherical. In deeper portions of the same tumor the cells had well marked queues or tails, or were of blunt spindle shape.

## PLATE 46.

FIG. 8. A section of the original sarcoma, showing tumor giant cells and infiltration of the skin.

FIG. 9. Invasion and replacement of liver tissue by sarcoma of the giant-celled type. *l*=columns of liver cells.

## PLATE 47.

FIG. 10. Hemorrhage from secondary nodules in the liver. The clot partially overlies the nodule from which the bleeding occurred. Some of the others are hemorrhagic.

## PLATE 48.

FIG. 11. Drawing of a minute metastasis in the liver, with a hemorrhage in its midst. *t*=tumor tissue; *h*=hemorrhage (nucleated erythrocytes).

## PLATE 49.

FIG. 12. Metastases in the ovary. The sarcomatous masses hang by pedicles like true ova.



FIG. 1.



FIG. 2.

(Rous and Murphy: Variations in Chicken Sarcoma.)





FIG. 3.

(Rous and Murphy: Variations in Chicken Sarcoma.)







FIG. 4.

(Rous and Murphy: Variations in Chicken Sarcoma.)



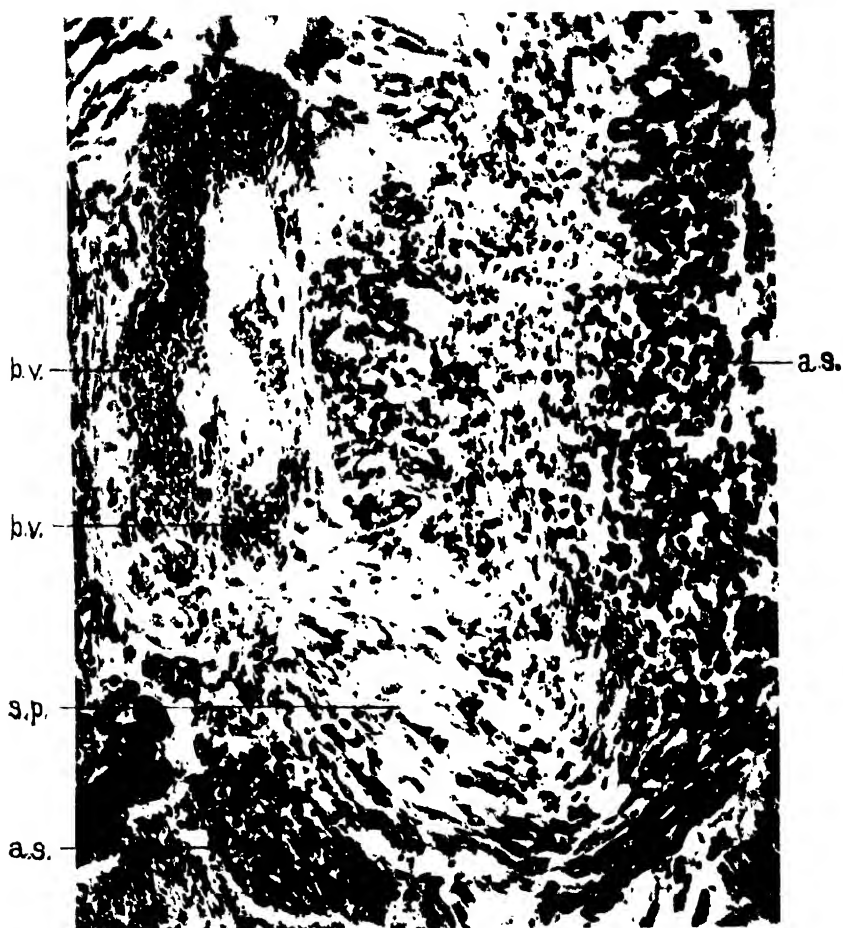


FIG. 5.

(Rous and Murphy: Variations in Chicken Sarcoma.)



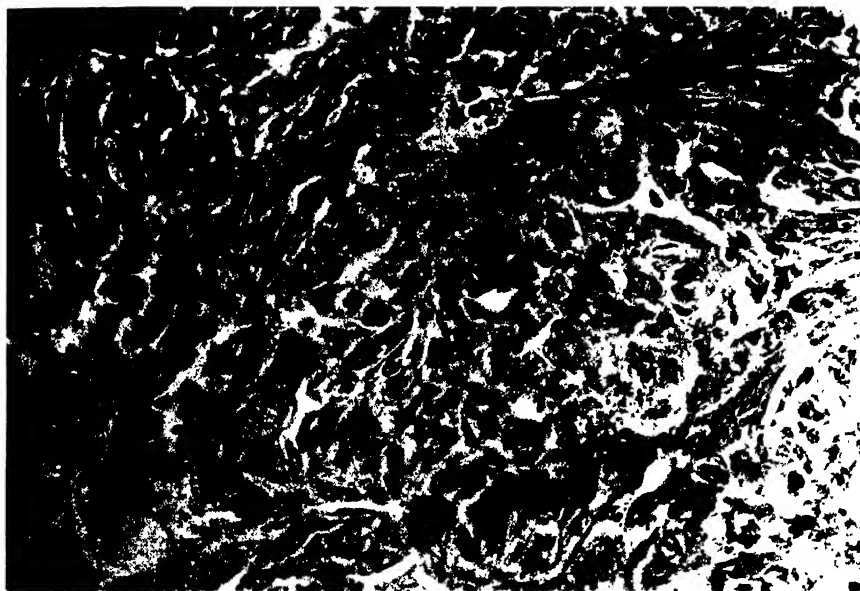


FIG. 6.

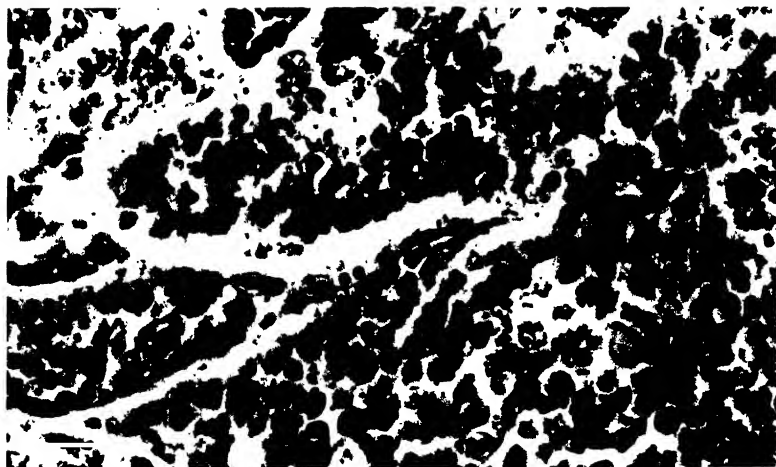


FIG. 7.

(Rous and Murphy: Variations in Chicken Sarcoma.)



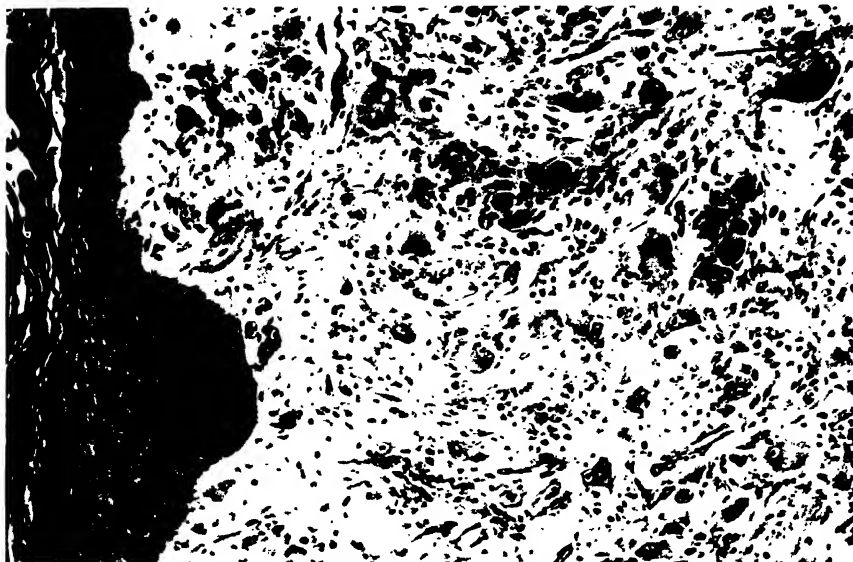


FIG. 8.

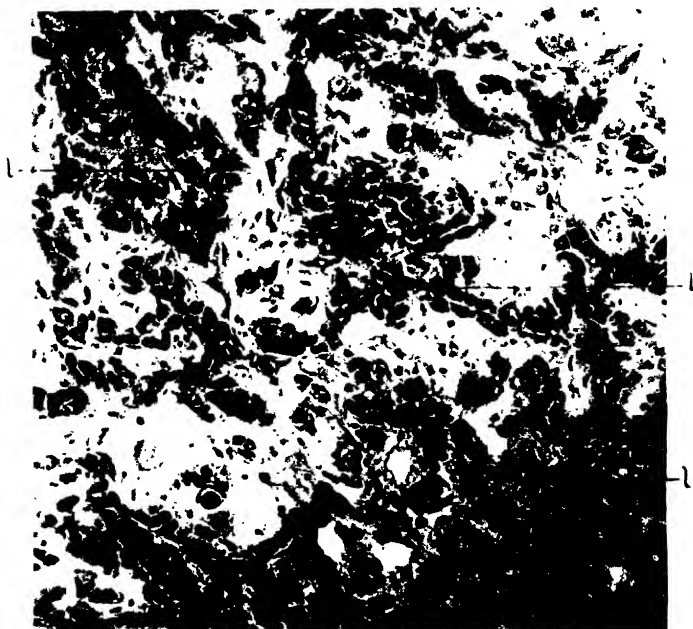


FIG. 9.

(Rous and Murphy: Variations in Chicken Sarcoma.)





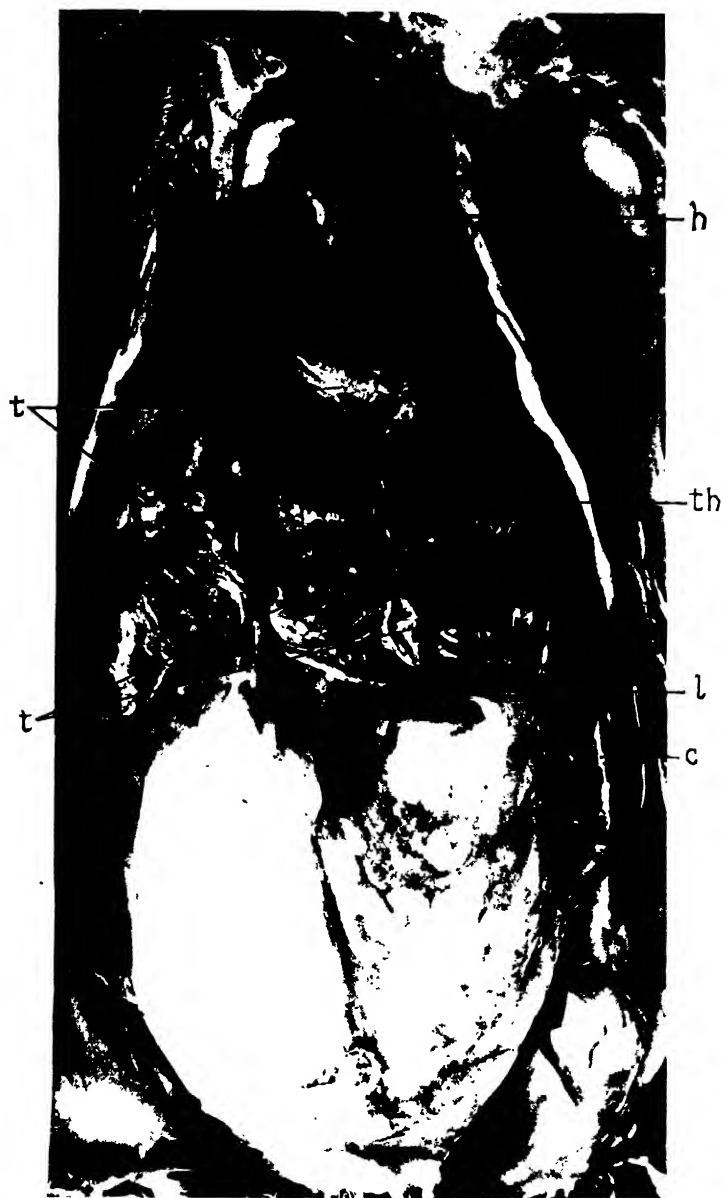


FIG. 10.

(Rous and Murphy: Variations in Chicken Sarcoma.)





FIG. 11.

(Rous and Murphy: Variations in Chicken Sarcoma.)





FIG. 12.

(Rous and Murphy: Variations in Chicken Sarcoma )



## A DEMONSTRATION OF TREPONEMA PALLIDUM IN THE BRAIN IN CASES OF GENERAL PARALYSIS.\*

BY HIDEYO NOGUCHI, M.D., AND J. W. MOORE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
and the Central Islip State Hospital, New York.)

### PLATE 50.

In the present communication we wish to report the results of examinations for *Treponema pallidum* on seventy parietic brains. One of us (Noguchi) succeeded in finding the pallidum in twelve out of the seventy specimens.<sup>1</sup>

The relationship of paresis to syphilis has, for years, been one of the foremost topics of medical interest, especially to psychiatrists. Since 1857 when attention was first called<sup>2</sup> to the frequency of syphilis in the history of patients with paresis, the etiological importance of the former disease has steadily gained recognition, until now probably the majority of writers agree with Kraepelin<sup>3</sup> that "We can to-day declare with the greatest certainty that syphilitic infection is an essential for the later appearance of paresis." Many, however, including a man of such enormous experience as Nonne, still refuse to concede that syphilis is more than an extremely common causative factor. Nonne<sup>4</sup> says, "At the outset I desire to make it clear that progressive paralysis is not a specific syphilitic disease of the brain."

Among those who hold "without syphilis, no paresis," there are some who contend that paresis is nothing more nor less than a particular form of tertiary syphilis. Kraepelin objects to this view

\* Received for publication, January 11, 1913.

<sup>1</sup> The findings were confirmed later by Dr. Moore as well as by others, among whom we may mention Dr. Flexner and Dr. Dunlap. We take pleasure in expressing our gratitude to Dr. G. A. Smith, Superintendent of the Central Islip State Hospital, for the material studied.

<sup>2</sup> Esmarch, F., und Jessen, W., *Allg. Ztschr. f. Psychiat.*, 1857, xiv, 20.

<sup>3</sup> Kraepelin, E., *Psychiatrie*, Leipzig, 1904, ii.

<sup>4</sup> Nonne, M., *Syphilis und Nervensystem*, Berlin, 1902.



on two principal grounds,—the distinctly greater interval between syphilitic infection and paresis as compared with that in cerebral syphilis and the refractoriness of paresis to antisyphilitic treatment. This author, however, in commenting on the hitherto vain attempts to find *Treponema pallidum* in the tissues and body fluids of paresis, remarks, "This does not mean that the spirochæta is never present in the body of the paretic. It may have assumed forms so far unknown to us or have located in places where it has not yet been sought or where it is hard to find."

Of late years a few cases have been reported in which the findings of paresis occurred along with those of cerebral syphilis. Sträussler<sup>5</sup> has recently added two cases of this combination to several he had already reported. Ranke<sup>6</sup> and others have demonstrated *Spirochæta pallida* in the pia and vessel sheaths in congenitally syphilitic brains. In this country Dunlap, of the New York State Psychiatric Institute, has also shown the organisms in a case of cerebral syphilis. Dunlap has long been of the opinion that, although tertiary syphilis of the brain and general paralysis are quite distinct and seldom, if ever, occur together, they are but different manifestations of the same process.

The failure up to the present time to discover *Treponema pallidum* in the affected nervous system has doubtless added to the general conception that paresis, although of syphilitic origin, can exist without the pallidum playing an active part in the progressive processes. This assumption is valid, of course, only when we grant that *Treponema pallidum* can infallibly be demonstrated by the microscopical techniques hitherto employed. But, as will be seen presently, this is far from being the case. Besides, the individual experience on the part of the examiner has much to do with whether the organisms are viewed or remain undetected, especially in a tissue in which so many tortuous fibers are present as in the brain.

These facts, coupled with the very significant serological similarities between syphilis and paresis, and the fact that the trypanosome

<sup>5</sup> Sträussler, *Ztschr. f. d. ges. Neurol. u. Psychiat.*, Orig., 1912, xii, 365.

<sup>6</sup> Ranke, *Ztschr. f. d. Erforsch. u. Behandl. d. jugendl. Schwachsinn*s, 1909, ii, 32, 81, 211.

can be found in the brain in sleeping-sickness,—a disease in many respects similar to paresis,—led the writers to believe that further search for the syphilitic organism, either in granular or in spirochætal form,<sup>7</sup> in paresis was warranted. To this end, tissues were taken from seventy paretic brains in the possession of the pathological service of the Central Islip State Hospital and stained with the Levaditi silver method modified in certain respects to produce an elective stain for the pallidum. The specimens in most of the cases were taken from the first right frontal gyrus; in some, from the corresponding area of the left hemisphere or gyrus rectus.

The syphilitic organisms were found in twelve cases which were examples of undoubted general paralysis. The cases in which the pallida were found showed the classical physical signs. The post-mortem findings in the brain in every case were definitely those of general paralysis. Ten were men and two were women. Seven were of the cerebral type, and five of the tabetic. In several instances the patients had been picked up on the street in a confused state and had no idea of the duration of the condition. In the seven cases in which the onset could be satisfactorily determined, the average duration was seventeen months, the longest thirty months, and the shortest five months. The majority were much below the average duration, as estimated by various authors, which varies from twenty-four to thirty-two months. It is possible, then, that we are more apt to find the spirochæta in those cases which run a fairly rapid course. The age varied from thirty-three to sixty years, with an average of forty-four years. Brief abstracts of the case records follow.

J. C. (103), age fifty years; duration of disease unknown. He was committed from the workhouse. There was much confusion; he was disoriented and his memory was extremely poor. The knee-jerks were unequal and exaggerated; the pupils sluggish and unequal. His speech was distorted, and there were marked tremors. Convulsions occurred. Death occurred after seven months in the hospital.

C. M. (106), age thirty-nine years; duration unknown, but probably about four months. Syphilis ten years previously. When he was admitted he was depressed and apprehensive; he had hallucinations of hearing; he was disoriented, and his memory was very poor, with spells of marked confusion. The

<sup>7</sup> Noguchi, H., *Jour. Am. Med. Assn.*, 1912, lix, 1236.

knee-jerks were normal, the pupils sluggish. There were ataxia, distorted speech, coarse tremors, and convulsions. He died after eleven months in the hospital.

F. B. (113), age thirty-six years; duration before admission, fourteen months. Syphilis was denied. On admission he was dull, silly, mildly restless, and had occasional hallucinations. His memory showed marked discrepancies. The knee-jerks were absent, and the pupils slow to light and unequal. His speech was distorted, and he had convulsions. Later he developed a silly elation and deteriorated progressively. Death occurred after fourteen months in the hospital. The total duration of the disease was thirty months.

S. V. (131), colored, age thirty-seven years; the duration of the disease before admission is unknown. Syphilis eight years previously. He was euphoric, his memory was very poor, and he was disoriented. The knee-jerks were much diminished, the pupils slightly slow, his speech ataxic, and there were coarse tremors. Later he developed typical expansive ideas and became very ataxic. Death occurred after twenty-seven months in the hospital.

J. D. (138), age thirty-three years; duration before admission, five months. He was admitted in an hallucinatory depression with nihilistic ideas and a history of a probable attempt at suicide. A complete mental status was impossible. The knee-jerks were exaggerated; pupils unequal, Argyll-Robertson sign present; there were marked tremors. The spinal fluid showed marked lymphocytosis. Death occurred after ten months in the hospital.

F. M. (169), age fifty-five years; duration of disease unknown. He was picked up on the street in a confused condition. He had absurd hypochondriacal ideas; "his insides were falling out," etc. He was disoriented and his memory was very poor. The knee-jerks were exaggerated, and the pupils sluggish. There were marked tremors, unsteadiness, and Romberg's sign. His speech was not seriously affected. Depression continued, and he insisted that he could not breathe or urinate, and that parts of him were dead. He died after four months in the hospital. The spinal fluid showed positive lymphocytosis and a positive Wassermann-Noguchi reaction.

E. W. (170), age forty-four years; duration unknown. He had had syphilis. The psychosis was a simple deterioration with marked confusion and disorientation but no delusions. He was silly, his memory was poor, and there were occasional attacks of excitement. The knee-jerks were diminished, and the pupils slightly slow. The speech showed extreme ataxia. Romberg's sign was present, and tremors were prominent. There were frequent convulsions. Death occurred after two months in the hospital.

E. R. (230), age forty-eight years; duration unknown. When admitted she was much demented, confused, completely disoriented, and her memory was poor. Her mood was one of silly elation. The knee-jerks were exaggerated, the pupillary reaction slow and of narrow range. There was marked distortion of speech and writing. There were coarse tremors and increasing ataxia. The spinal fluid showed marked pleocytosis. Death occurred after six months in the hospital.

M. M. (235), age forty-two years; duration uncertain. She had been blind from optic atrophy for two years before admission and had experienced occasional visual hallucinations, but definite mental symptoms seem to have begun only a few weeks before commitment, when she began to show a change of disposition and became forgetful. Syphilis probably occurred twenty years previously. Her husband was a paretic. On admission she was excited, resisting, and obscene. She seemed clear and gave correct answers in the orientation test but her memory showed marked discrepancies in time relations. The knee-jerks were absent, the pupils rigid, her speech drawing and distorted, no tremors, and only slight ataxia. The spinal fluid showed extreme lymphocytosis. She quieted down, and during the next two years showed a gradual deterioration without any particular mental trend. Ataxia increased steadily; she had occasional apoplectic attacks and convulsions. Death occurred after two years in the hospital.

G. F. (236), age sixty years; duration before admission, one month. He became gradually childish, forgetful, and irritable. When admitted he was disoriented, and his memory was poor. The knee-jerks were absent, and the pupils unequal and slow; his speech was slurring and distorted. There were marked tremors and Romberg's sign. He deteriorated rapidly and died after four months in the hospital.

M. D. (242), age thirty-seven years; duration eight months. The onset seemed to date from an injury to the head. There was a probable history of syphilis. He was depressed, confused, and had attempted suicide. His memory was poor, and he was much demented. The knee-jerks were exaggerated; and the pupils unequal and Argyll-Robertson sign present; his speech was ataxic. There were tremors and occasional convulsions. He died after four months in the hospital. The total duration of the disease was one year.

F. B. (299) (figure 1), age fifty-eight years; duration before admission, one month. There was a probable history of syphilis. He was depressed and whining, and had typical paretic ideas, such as that his bowels never moved, and that he had an incurable disease. He would point to a leg or an arm and say, "See, it's all dead, all gone,—there's no hope." His orientation and memory showed marked defects. The knee-jerks were slightly exaggerated, the pupils slow, his speech was distorted, and tremors were excessive. The spinal fluid showed pronounced lymphocytosis and a positive Wassermann-Noguchi reaction. Death occurred after eleven months in the hospital.

Naturally the first question that arises is, Are these not cases of cerebral syphilis in the narrower sense? In reply to this we can but review briefly the differential points. They all showed, in their clinical course, a diffuse, progressive deterioration in all the mental fields, and not the *démence lacunaire* so often observed in syphilitic brain disease. There were no cranial symptoms or other focal manifestations. These points are admittedly not convincing, for it is possible for an old syphilitic endarteritic-meningitic condition to

simulate paresis so closely as to be indistinguishable by its clinical features from the latter disease. It is upon the post-mortem findings that the diagnosis must depend. Anatomically the brains of our cases showed the usual pial thickening, more marked over the frontal convexity. In two it was also prominent over the gyri recti, cerebellum, and cisterna, but it did not assume the degree of meningitis usually seen in syphilis of the base. In one case (235) there was an old hemorrhagic membrane beneath the dura; in another (131) there was a marked preponderance of the parietic process in the right hemisphere, but these conditions were not represented clinically and the findings were otherwise typical. In the microscopic examination the meningeal process in all cases was diffuse, being more marked in the frontal region. The vessel infiltration was also diffuse and found at all depths of the cortex and in the marrow. Plasma cells were, in each case, numerous and usually outnumbered the lymphocytes. Rod cells were always present. The nerve cell alterations and changes in the neuroglia do not present differential characteristics and will not be enumerated. With regard to the vessels, a number of our cases, as in all general paralysis material, showed endarteritic changes. In six there was definite thickening of the vessel walls of the type described as syphilitic. In no instance did the intimal proliferation embarrass the lumen to any extent. In the remaining cases the vessels appeared normal. It seems hardly possible that the presence of the spirochætæ could be traced to the luetic endarteritis, since they were not found associated, at least with those vessels which are visible with the Levaditi stain. In none of the cases were there softenings, and no gummata were found either in gross or microscopically.

The spirochætæ were found in all layers of the cortex with the exception of the outer, or neuroglia layer. One was located at the border of this layer, but not within it. A few were found subcortically. Careful search of the pia failed to reveal any of the organisms. In all instances they seemed to have wandered into the nerve tissue. They were not found in the vessel sheaths and seldom in close proximity to the larger vessels. There seems to be no ratio between the number of spirochætæ and the severity of the

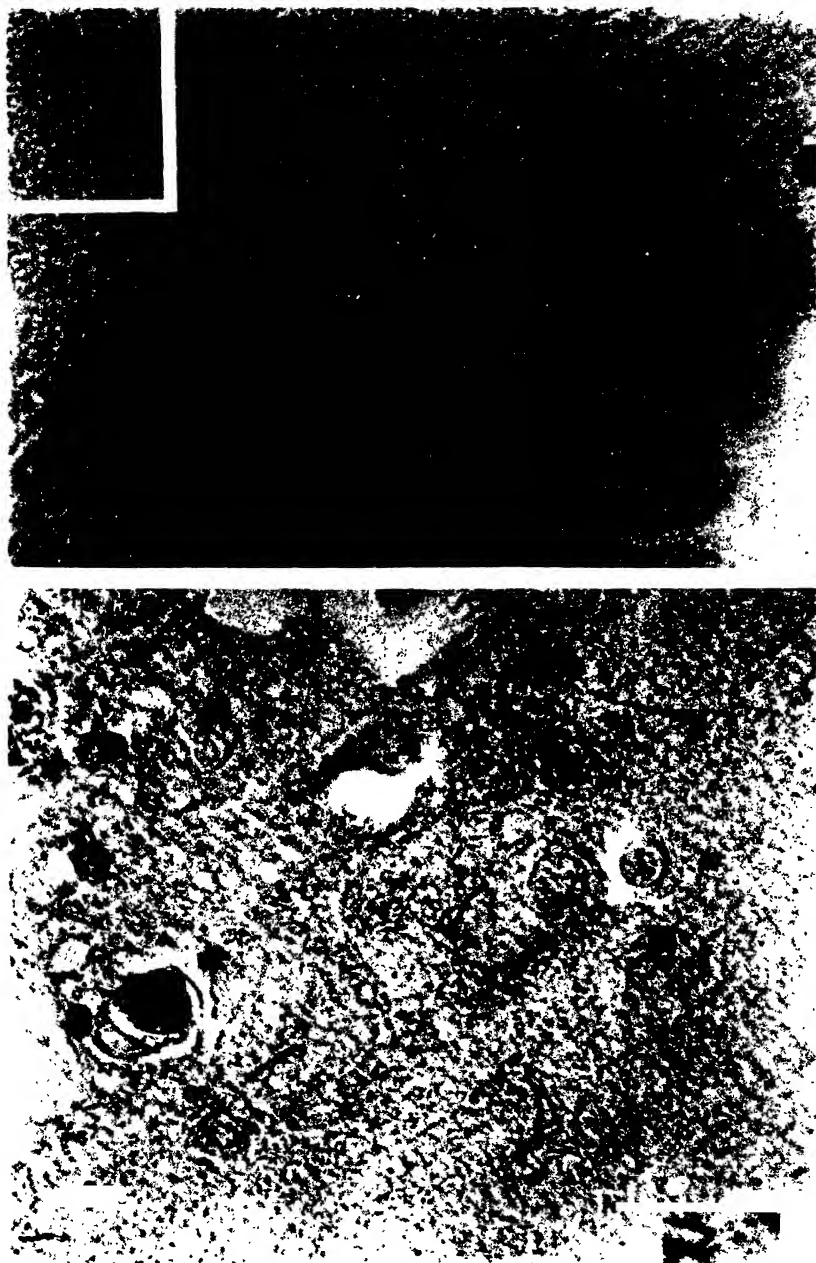


FIG. 1.

(Noguchi and Moore: *Treponema pallidum* in the Brain of General Paralysis.)



paretic process, although the case in which they were most numerous showed excessive paretic changes.

Whether or not, by improving the technique, *Treponema pallidum* can be demonstrated in a much higher percentage of paretic cases will be determined by further investigations.

#### EXPLANATION OF PLATE 50.

FIG. 1. *Treponema pallidum* in the cortical layer of the right frontal area of the brain (case 299). Stained, with slight modification, by Levaditi's method.  $\times 1,100$ .



## THE REACTION BETWEEN OXYGEN AND HEMOGLOBIN.

By E. E. BUTTERFIELD.

*(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)*

There are at present four different views as to the nature of the absorption of oxygen by blood.

The first and oldest view is expressed by the relation,

1 mol. hemoglobin + 1 mol. oxygen  $\rightleftharpoons$  1 mol. oxyhemoglobin.

According to the law of mass action one would have

$$ab = kc, \quad (1)$$

in which  $a$  represents the concentration of hemoglobin in solution,  $b$  the oxygen concentration,  $c$  the concentration of oxyhemoglobin, and  $k$  a constant.

The second view is that of Bohr. Bohr assumed that hemoglobin in aqueous solution is hydrolytically split into globin + Fe-component, and that the Fe-component combines reversibly with oxygen. Bohr's formulation of this hypothesis leads to an equation of the fourth degree. Without going into details it will suffice to call attention to the main points of Bohr's work as far as we are concerned here. They are, in addition to the assumed hydrolysis of hemoglobin in aqueous solution, first, the inapplicability of formula (1) to his results and secondly, the variation of the ratio  $\frac{\text{oxygen absorbed}}{\text{total hemoglobin concentration}}$  with the total hemoglobin concentration<sup>1</sup> at constant pressure:

<sup>1</sup> By total hemoglobin concentration is meant here and in the following the total weight of blood coloring matter (hemoglobin + oxyhemoglobin) in unit volume.

The third view is that of Wo. Ostwald who applied the older adsorption formula,

$$x = kc^n, \quad (2)$$

in which  $x$  = oxygen adsorbed,  $c$  = oxygen concentration,  $k$  and  $n$  are constants, to some of the data of Paul Bert and Loewy and found that  $\log x$  and  $\log c$  plotted in rectangular coordinates gave a straight line.

The fourth and last view is that brought forward by Manchot, who found that the quantity of oxygen or CO absorbed by 100 c.c. blood, laked or unlaked, varied with the dilution, *i. e.*, with the total hemoglobin concentration. On 10-fold dilution this quantity approached a maximum value of 2 mol. oxygen to 1 mol. hemoglobin.

The experiments which I have to report were carried out during the last 4 years in the course of other studies. These experiments are restricted to the influence of change in the total hemoglobin concentration on the amount of gas bound pro gram hemoglobin at constant gas pressure. The results also hold only for hemoglobin in solution, *i. e.*, aqueous solutions of crystalline oxyhemoglobin or centrifuged red corpuscles dissolved in  $H_2O$ . It is to be emphasized that the few experiments cited here have been selected solely with reference to the lowest and highest total hemoglobin concentration in each series. Four entirely different methods were employed and they all lead to the same result.

TABLE I.

*Absorption of CO. Hemoglobin Spectrophotometrically.*

	$\rho$	$c$	$\frac{CO}{Hb}$	
I.	654	3.59	1.29	Laked ox blood.
	658	4.86	1.30	
	668	2.88	1.29	Laked human blood.
	616	5.40	1.27	

*Reduction with Palladium-Hydrogen. Hemoglobin Gravimetrically.*

	$\rho$	$c$	$\frac{O_2}{Hb}$	
II.	156	1.23	1.09	Oxyhemoglobin from ox blood.
	154	11.50	1.08	

*Oxygen Capacity by Ferricyanide Method. Hemoglobin Spectrophotometrically.*

	<i>c</i>	$\frac{O_2}{Hb}$	
III.	1.64	1.37	
	4.27	1.40	Laked rabbit blood.

*Spectrophotometric.*

	Dilution	$\frac{\log \frac{I_1}{I_1'}}{\log \frac{I_2}{I_2'}}$	
	2	1.12	
	10	1.13	Laked ox blood.
	1.5	1.13	Laked ox blood after extraction
	10	1.13	with ether.
IV.	200	1.13	

---

	<i>c</i>	$\frac{\log \frac{I_1}{I_1'}}{\log \frac{I_2}{I_2'}}$	
	1.14	1.13	
	7.00	1.13	Oxyhemoglobin from ox blood.

The first group of experiments was carried out with laked ox blood and with laked human blood. The volume of CO absorbed by oxygen-free blood ( $(NH_2)_2 \cdot H_2O$  and vacuum pump) was determined. The quantity of CO physically absorbed was deducted from the total volume, using the coefficient of absorption for CO in  $H_2O$  at the temperature of the experiment ( $20^\circ C.$ ) for the calculation. Total hemoglobin concentration determined spectrophotometrically. It will be seen from the table that for a small range of concentration (2-fold) the CO absorption pro gram hemoglobin is practically constant for laked ox blood or human blood. (In the table,  $c$  = hemoglobin concentration, gram in 100 c.c., CO = volume CO absorbed by hemoglobin, Hb. = total hemoglobin quantity in grams, and  $p$  = pressure of CO in mm.) The deviations are no greater than could be accounted for by the slight decrease of the solubility of CO in hemoglobin solutions of ascending concentrations. It will be noticed that at a CO pressure of 650 mm. the limit 1 mol. CO to 1 mol. hemoglobin is almost reached (theor. 1.34 c.c. CO pro gram hemoglobin). The difference again is in all probability due to the difference in coefficients of absorption of CO in  $H_2O$  and in blood of the concentrations studied. The reason for the good agreement

between the values for ox blood and human blood rests in the identity of the optical constants for human hemoglobin and ox hemoglobin.

The second series of results was obtained by the method of Paal. Reduction of dialyzed solution of oxyhemoglobin, saturated with air at the prevailing atmospheric pressure, in an atmosphere of hydrogen in the presence of colloidal palladium. The quantity of hydrogen absorbed was measured. It was assumed that under the conditions of the experiment  $\text{oxyhemoglobin} + \text{H}_2 = \text{H}_2\text{O} + \text{hemoglobin}$ . Previously the quantity of  $\text{H}_2$  absorbed by 0.1 gram Pd in 110 c.c.  $\text{H}_2\text{O}$  was determined. This was deducted from the total  $\text{H}_2$  absorbed in the experiments with hemoglobin. In all of these experiments 0.1 gram Pd and 110 c.c. solution were constantly used. Hemoglobin concentration determined gravimetrically. It will be seen from the table that for an almost 10-fold variation in the hemoglobin concentration the quantity of  $\text{O}_2$  pro gram Hb. remains constant. The value is considerably lower than for CO at high pressures. It is not necessary to go into the various possibilities (incomplete reaction, adsorption of hemoglobin by Pd, etc.) which may account for this difference. It suffices here to call attention to the fact that the quantity of  $\text{O}_2$  given off pro gram Hb. to a reducing agent is independent of the total hemoglobin concentration if the solutions have been previously saturated at the same partial pressure of oxygen.

The third series<sup>1</sup> was obtained by the ferricyanide method of Haldane and Barcroft with laked rabbit blood saturated with oxygen at the prevailing partial pressure in the air. Total hemoglobin concentration determined spectrophotometrically, using the constants of oxyhemoglobin from ox blood. Here again for a 3-fold variation in concentration  $\text{O}_2/\text{Hb.}$  is constant. The apparent slight excess over 1 mol.  $\text{O}_2$  to 1 mol. Hb. is probably due to a slight difference in the optical constants of oxyhemoglobin from rabbit blood and oxyhemoglobin from ox blood.

The fourth and last series was obtained from measurements of the light absorption of laked ox blood and oxyhemoglobin from ox

<sup>1</sup> From an unpublished study on the action of pneumococcus on blood, by Dr. F. W. Peabody and myself.

blood at the wave-lengths 577, 579  $\mu\mu$  (double line, mercury arc) and 546  $\mu\mu$ .  $\log (I_1/I_1')$  is the log of the ratio of initial and final intensities in the solution at 577, 579  $\mu\mu$ ,  $\log (I_2/I_2')$  the log of the corresponding ratio at 546  $\mu\mu$ . Between the quotient of the logs of these ratios and the concentration of hemoglobin (oxygen-free) in the presence of oxyhemoglobin there exists the following relation:

$$\frac{\log \frac{I_1}{I_1'} (\beta_1 - \alpha_1)x + \alpha_1}{\log \frac{I_2}{I_2'} (\beta_2 - \alpha_2)x + \alpha_2}, \quad (3)$$

in which  $x$  = relative concentration of hemoglobin,  $\alpha_1, \alpha_2, \beta_1, \beta_2$

constants which can be determined experimentally. If  $\frac{\log \frac{I_1}{I_1'}}{\log \frac{I_2}{I_2'}}$

remains constant on dilution then  $x$  must be constant. That is if

$\frac{\log \frac{I_1}{I_1'}}{\log \frac{I_2}{I_2'}}$  remains constant on dilution at constant  $O_2$  pressure the

relative composition of the solution (oxyhemoglobin and hemoglobin) also remains constant.

We will now apply these results to the existing views on the nature of the absorption of oxygen by hemoglobin. Several considerations not embodied in the table will also be of aid in selecting the most probable formula.

The Bohr formula falls out for the following reasons:

1. When the globin is split off from the hemoglobin molecule the iron-component can be identified as hematin (or its reduction product, hemochromogen), which has a characteristic spectrum. All of our spectroscopic and spectrophotometric evidence is against the presence of hematin or hemochromogen in normal blood or in solutions of freshly prepared oxyhemoglobin. I have recently been able to split hemoglobin in glycerin solution by means of  $(NH_2)_2 \cdot H_2O$  reversibly into globin + hemochromogen. The re-

action, however, only takes place under special conditions and is always accompanied by sharp spectral changes.

2. Granted for the moment that hemoglobin is hydrolyzed in aqueous solution. Then it must be possible to shift the equilibrium by changing the concentration of  $H^+$  or  $OH^+$  ions. Following experiments<sup>1</sup> show that such a shift of equilibrium does not occur or at least if it does it has no influence on the combination of oxygen with hemoglobin at constant pressure. Similar results were obtained with KOH. On increasing the  $H^+$  or  $OH^+$  concentration beyond the quantities given in the table a constitutive change occurs in the oxyhemoglobin molecule; methemoglobin is formed.

TABLE II.

	<i>c</i>	O <sub>2</sub> -Capacity.	O <sub>2</sub> Hb	Quantity of $\pi/10$ HCl Added to 4.5 c.c. Washed Rabbit Corpuscles in c.c.
I	4.27	5.97	1.40	0.0
II	4.47	6.25	1.40	0.5
III	4.48	6.37	1.42	1.0
IV	4.40	6.35	1.44	2.0

3. From Bohr's experiments and formula the quantity of O<sub>2</sub> absorbed pro gram hemoglobin is a function of the total hemoglobin concentration. According to the experiments here presented the quantity of O<sub>2</sub> bound pro gram hemoglobin is independent of the total hemoglobin concentration at constant gas pressure.

The arguments 2 and 3 against the Bohr formula also hold for Manchot's work. In addition it is to be noted that in our experiments the limit value for the oxygen (or CO) absorption is 1 mol. oxygen to 1 mol. hemoglobin. In no case does it approach 2 mol. The spectrophotometric data are conclusively against Manchot's views. Manchot reached the conclusion that undiluted blood contains about 33 per cent. hemoglobin uncombined with oxygen, while at 10-fold dilution the amount of uncombined hemoglobin approaches zero. From the spectrophotometric data it follows that  $x$  (or  $100x$ , the percentage of uncombined hemoglobin) is practically independent of dilution at constant oxygen pressure.

Of the four views mentioned in the beginning, this leaves the choice between the mass action formula for a monomolecular chem-

<sup>1</sup> From the pneumococcus study by Dr. Peabody and myself.

ical reaction and the adsorption formula. Our experimental results furnish little aid in this choice, except the fact that the limit value of oxygen (or CO) absorption is 1 mol. gas for each mol. hemoglobin. This would indicate, of course, a monomolecular reaction. A very strong argument against the adsorption theory is the fact that combination of hemoglobin with oxygen is accompanied by a sharp change in the spectrum, which as far as I know can only be interpreted as constitutive molecular change. Not having any data of my own suitable for a test of the adsorption formula, I have taken the data of Bohr, Hüfner, and Loewy. In no case is  $n \log c + \log k$  a straight line function of  $\log x$ . Even from Loewy's numerical data it is impossible to find support for the adsorption theory—it will be recalled that Wo. Ostwald bases his claim for the adsorption of oxygen by blood chiefly on a curve of Loewy's. The formula  $x = kc^n$  is only of limited application to adsorption phenomena. On trying the more general formula of Arrhenius on the same data one finds that  $k$  is not constant. So on the whole it may be said that there is no evidence to support the view that oxygen is *adsorbed* by hemoglobin. It is possible that adsorption may play a rôle in the taking up of oxygen by intact red corpuscles, but there too the chief phenomenon is more probably the chemical combination of oxygen with hemoglobin.

This leaves finally the first and oldest view of reaction between oxygen and hemoglobin as the only one of the four which is at the same time tenable and compatible with the results here presented. These results are entirely in accord with formula (1),  $b$  being practically constant in each series (or varying slightly with the solubility of the gas in solutions of different total hemoglobin concentration). We would have then,

$$\frac{\text{conc. hemoglobin}}{\text{conc. oxyhemoglobin}} = \text{const.},$$

which is a uniform result at constant pressure and temperature, obtained by four different methods. It is necessary in closing to call attention to the fact that in all experiments the gas pressures were relatively high and that under these conditions one is working in the neighborhood of maximum saturation of hemoglobin with CO or oxygen.

## THE EXTRACELLULAR RELATION OF THE MALARIAL PARASITE TO THE RED CORPUSCLE, AND ITS METHOD OF SECURING ATTACHMENT TO THE EXTERNAL SURFACE OF THE RED CORPUSCLE.\*

By MARY ROWLEY-LAWSON, M.D.

(From the Laboratory of Dr. Mary Rowley-Lawson, New London.)

### PLATES 56 TO 61.

Since the malarial parasite was first described in 1880, many views have been held as to the morphology of the ring-form parasite, and its possible formation and significance, but with a remarkable similarity of opinion among observers as to the parasite being intracellular.

Laveran (1) has consistently claimed from the beginning that the parasites were extracellular and that they attached themselves to the surface of the red corpuscles, adhering to them "by pressing on them."

Richard (2) thought at one time that the parasites were within the substance of the red corpuscle; but later (3) he returned to Laveran's opinion that they were attached to the surface.

Mannaberg (4) inclines to the opinion that the young unpigmented parasites "lie for a considerable time upon the blood corpuscles. . . . In the places where the parasites were observed, distinct impressions in the substance of the blood corpuscles are noticed, and that the margin of the so-formed depression is perfectly sharp. . . . If these bodies were within the substance of the blood corpuscle, one cannot understand how the observed depression in the upper surface or edge of the blood corpuscle takes place."

Marchiafava and Bignami (5) describe a similar condition, depicting the parasites as being embedded or pressed into the surface of the red corpuscle; but these observers believe the greater number of parasites, even the young forms, to be endoglobular.

When Koch was in this country he was shown models of the red corpuscles with the malarial parasites beneath the surface, and he made the statement that the parasites were not within the substance of the red corpuscle but attached to the surface.

\* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, December 16, 1912.



THE METHOD BY WHICH THE MALARIAL PARASITE SECURES  
ATTACHMENT TO THE OUTSIDE OF THE RED CORPUSCLE.

The malarial parasite is extracellular during its entire developmental cycle; that is, with the exception of the brief periods when it is free in the blood serum, it is attached to the outside surface of the red corpuscle.<sup>1</sup>

The parasite secures its attachment to the outside of the red corpuscle by means of filamentous pseudopodia thrown out by the parasite for that purpose. The attaching processes will be described as (1) primary and (2) secondary (accessory) attaching pseudopodia.

(1) *Primary Attaching Pseudopodia*.—(Plates 56 to 61). These are delicate thread-like processes, arising from the cytoplasm of the parasite, near its nucleus (plate 56, figure 2; plate 59, figure 1). The parasite appears to secure its primary hold on the red corpuscle by means of these pseudopodia; but as the corpuscular mound to which the parasite is attached becomes dehemoglobinized, or decolorized, or as the parasite increases in size, accessory, or secondary attaching pseudopodia are formed.

(2) *Secondary Attaching Pseudopodia*.—These may be thrown out from various portions of the cytoplasm of the parasite, apparently for the purpose of securing a firmer hold on the red corpuscle (plate 58, figure 7, x; plate 59, figure 15, x).<sup>2</sup> The pseudopodia can be seen overlying the red corpuscle in the form of a loop, while the body of the parasite, with its nucleus, may lie either on the surface of the red corpuscle (plate 58, figures 1 and 2, and 4 to 10) or along its periphery in close apposition to the corpuscle (plate 56, figures 6 to 11). Occasionally, especially in the very young parasite, the loop may be formed from the entire protoplasm of the parasite (plate 59, figure 9).

The parasite squeezes up that portion of hemoglobin substance which lies within the boundary of this loop into a mound, circular at the base, with a more or less rounded apex (plates 56 to 59). The

<sup>1</sup> The red corpuscle will be described in this article as having an upper and an under surface, and a periphery.

<sup>2</sup> The accessory filaments are especially common to the tertian parasite, even in the earliest stages of its development.

parasite when thus attached maintains its position on the outside of the red corpuscle by means of these pseudopodia encircling the mound of corpuscular substance at its base.

When viewed from above the young parasites with their pseudopodia encircling mounds of corpuscular substance give the characteristic ring-form pictures (plate 56, figures 7 to 10; plate 58, figures 1 and 2, 4 to 10, and 12 and 13; plate 59, figures 9 and 10; plate 60, figures 1 to 4), which have been so variously interpreted. This interpretation of the ring-form parasite explains the absence of pigment in the corpuscular mound area and makes it clear why it has been found impossible to stain this area.

The true interpretation of these ring-forms might be difficult if the hemoglobin mounds were not seen projecting outward from the periphery of the red corpuscle, surrounded by the parasites (plate 56, figures 13 to 35; plate 57, figures 1 to 23, 27 and 35; plate 58, figures 3, 11, 14, and 26 to 29; plate 59, figures 2, 4, and 11 to 21; plate 61, figures 5 to 9, and 11). But the fact that they are seen in this position makes the interpretation easy, especially as the delicate, thread-like pseudopodia can be seen encircling the hemoglobin mound. Very often the encircling pseudopodium can be traced in its entirety around these peripheral mounds (plate 56, figures 18 to 20, and 29; plate 57, figures 16, 32, and 35; plate 58, figures 26 and 27).

Occasionally a part of the encircling filament is obscured by the thickened mound of corpuscular substance overlying it, this being due to the fact that the mound has been flattened or pressed down in the process of smearing the blood (plate 57, figures 26 to 28, and 30 and 33; plate 58, figures 4 to 6); but careful focussing generally allows even these portions of the pseudopodium to be seen. When studying the parasites in fresh blood preparations, portions of parasites lying beneath these pressed down mounds of corpuscular substance might lead one to think that the parasites were within the substance of the red corpuscle.

With very few exceptions, the adult parasite is attached to the surface rather than to the periphery (plate 60, figures 13 and 14) of the red corpuscle. I believe that the explanation of this is that the surface of the red corpuscle affords a larger area for occupation

as well as for absorption. In this situation, moreover, the chances for the forcible removal of the parasite from the red corpuscle are fewer.

When the parasite is attached to the under surface of the red corpuscle (plate 58, figure 14, A) the blue staining protoplasm of the parasite viewed through the red corpuscle is paler and the outlines of the parasite are less distinct than when the parasite is seen on the upper surface of the red corpuscle (plate 58, figure 14, O). When two parasites are attached to the one corpuscle, one on either surface, these differences can be well seen (plate 58, figures 14 and 26). Also the same differences can be noted when a single parasite is seen on both surfaces of the red corpuscle (plate 57, figures 7, 14, and 22; plate 59, figures 4 and 5).

When a parasite, especially an adult, is attached to the under surface of a red corpuscle, the nucleus may be indistinct or obscured from view by the overlying, blue-stained protoplasm of the parasite, and may give the impression of a parasite without a nucleus. And the nucleus of a free parasite may be hidden from view, if it is on the under surface of the parasite.

Occasionally an adult parasite is attached to a corpuscular mound in a position so close to the periphery of the red corpuscle that the nucleus of the parasite projects beyond the periphery (see plate 60, figure 15, where the nucleus of the parasite extends beyond the periphery of the corpuscle to which the parasite is attached, and overlies the red corpuscle adjoining the infected one). It is not at all uncommon to see the nucleus of the young parasite of the æstivo-autumnal infections projecting beyond the periphery of the red corpuscle to which the parasite is attached (plate 56, figures 13 and 32; plate 57, figures 12 and 15; plate 58, figures 6 and 7), and occasionally the nucleus of a tertian ring may be seen beyond the periphery of the red corpuscle (plate 59, figure 7). The blue-stained protoplasm of a parasite may also be seen beyond the periphery of the corpuscle to which the parasite is attached (plate 56, figures 6 to 12, and 14 to 17, and 22; plate 58, figure 25; plate 59, figure 3).

Ewing (6) has noted the fact that portions of a parasite may go beyond the periphery of the corpuscle to which the parasite is attached: "In many tertian

cases, the body and especially the nucleus of the parasite appear to project beyond the border of the cell, even more distinctly than in the case of the aestivo-autumnal ring."

Often when a parasite has attached itself to a corpuscular mound, that portion of the nucleus of the parasite which is next to the mound may be seen to have conformed itself to the circular shape of the mound (plate 57, figures 34 and 35).

*Retraction of Hemoglobin.*—This is a condition caused, I believe, by the parasite which, in the process of pulling up the corpuscular mound for the purpose of attaching itself to the red corpuscle, draws up a portion of the hemoglobin from the rest of the corpuscle in such a manner as to leave decolorized areas at the periphery of the corpuscle, the contour of the corpuscle remaining undisturbed (plate 57, figures 11, A, and 12, A).

In discussing this retraction of the hemoglobin, Thayer and Hewetson (7) state: "In some cases we may see corpuscles containing small, refractive, *ring-like* bodies, with or without pigment, where the hemoglobin seems to have left the outer part decolorized though still showing the outline of the original corpuscle." Craig (8) has also noted these colorless areas, describing the condition as "retraction of hemoglobin."

*The Formation of Rings by the Joining of Pseudopodia.*—This has been noted by several observers.

Thayer and Hewetson (9) have observed that a small parasite may form a ring through the meeting and fusing of pseudopodia, and noted that in the middle of this ring there lies, apparently, a bit of corpuscular substance. Thayer (10) states that "some observers believe that such a portion of the corpuscle included within two pseudopodia is gradually digested by the parasite."

DaCosta (11) writes that rarely the ring may be formed by the fusion of two short pseudopodia between which a small portion of the corpuscle may become imprisoned, and Ewing (12) states that "secondary" (accessory) rings sometimes form from the union of pseudopodia, and that these are identical in appearance with the primary ring, but lack the chromatin granules." Osler, Councilman, and Antolisei (13) believed that some of the ring-forms were small parasites with portions of the red corpuscles included within their substance. Marchiafava and Bignami (14) do not deny that there are parasites which have included a portion of the red corpuscle, but they believe it to be an atypical process.

In view of these facts Mannaberg's (15) observations are interesting: "If such a ring is observed with an open Abbe and oblique illumination with a concave mirror, it is definitely seen to produce in the upper surface of the blood

corpuscle a deep and sharp depression, and in the center the substance of the blood corpuscle projects through the hoop just as a finger through a ring."

**THE DECOLORIZED CORPUSCULAR MOUND, OFTEN CALLED THE  
ACHROMATIC AREA OR MILKY ZONE.**

The terms achromatic area, or milky zone, have been applied to the decolorized, transparent, and pigment-free corpuscular area encircled by the filamentous pseudopodium of the parasite.

The decolorization of the corpuscular mound is gradual, proceeding as the parasite digests the hemoglobin. All stages between the deeply colored mound of unaltered hemoglobin and the completely decolorized one have been observed and can be followed with the microscope in fresh blood as well as in stained specimens (stages between A and O, plate 58, figure 7).

Complete decolorization, or dehemoglobinization, of corpuscular mounds surrounded by parasites may be seen in red corpuscles showing Schuffner's granulation (plate 60, figure 1), and in red corpuscles with blue stippling (plate 60, figure 4), suggesting that these granulations form a part of the corpuscular substance digested by the parasite.

The decolorization of the corpuscular mound by the young ring-form parasite, especially of the æstivo-autumnal infections, may be complete before the appearance of any demonstrable pigment in the protoplasm of the parasite.

It has been believed that the achromatic area was an essential part of the malarial parasite, and it has been variously described as a vesicular nucleus, a digestive vacuole, and a thinning of the protoplasm of the parasite which allowed the red corpuscle to show through. When certain of these decolorized areas were seen at the periphery of the red corpuscle (plate 57, figures 24 and 25; plate 58, figures 12 and 13), they were pictured and described by Manna-berg and Marchiafava and Bignami, who interpreted them as extra-cellular parasites resting in niches at the periphery of the red corpuscle.

That the colorless, or dehemoglobinized, corpuscular area surrounded by the parasite is more or less transparent seems proven by the following facts: (a) When the achromatic area of one corpuscle

partially overlies the periphery of another red corpuscle, this periphery can be distinctly seen through the achromatic area (plate 58, figure 12). (b) When the achromatic area of one red corpuscle partially overlies that of another red corpuscle, the nucleus and the protoplasm of the parasite surrounding the underneath decolorized area is clearly seen (plate 58, figure 13). (c) When the smear is made on the cover-slip, precipitation of stain may be seen more or less clearly through the decolorized corpuscular mound. (d) When the parasite-infected corpuscles are piled up, one completely over another, it is impossible to find any completely decolorized corpuscular mounds, although many decolorized areas surrounded by parasites may be seen where the smear is thinly spread.

The fact that the area which is surrounded by the parasite is sometimes seen to be achromatic and transparent, when other portions of the infected corpuscle show good color, proves that this area is not parasitic. For if this area belonged to a parasite which was attached to a deeply colored red corpuscle (plate 58, figures 12 and 13), the area would have to be opaque in order to be achromatic, otherwise the color of the corpuscle would show through it more or less clearly.

The gradual decolorization of the corpuscular mound undoubtedly has given rise to many conceptions regarding the finer structure of the ring-form parasite.

For example, many observers believe the rings to be ameboid bodies, which, becoming very thin at the center or being palely stained or unstained, permit the corpuscle to show through, thus giving the parasite the appearance of being ring-shaped. I believe this view is held by Marchiafava and Celli (16), Celli and Guarnieri (17), Mannaberg (18), Thayer and Hewetson (19), Bastienelli and Bignami (20), Ziemann (21), and Opie (22), and it is the view formerly held by the author (23).

Argutinsky (24) interpreted the ring-form parasites as artefacts, and Nocht (25) believed them to be optical illusions due to discoid bodies with thickened rims.

Others held the view that the achromatic area was a bladder-like nucleus in which lies a small, deeply staining chromatin mass. Among those who incline to this view, are Sakharoff (26), Canalis (27), Celli and Sanfelice (28), Grassi and Feletti (29), Romanovsky (30), Manson (31), Osler (32), Cabot (33), and Craig (34).

Still other observers, Marchiafava and Bignami (35), Schaudinn (36), Maurer (37), Calkins (38), Loeffler (39), and others, believe the achromatic area, or decolorized corpuscular area, to be a nutrition vacuole.

In depicting the changes in color of the corpuscular mound, Marchiafava and Bignami (40) state: "The ring surrounds a space which takes the same stain as the red corpuscle, especially in its youngest stage, while in the center of more developed forms the red corpuscle is of paler appearance than it is outside of the ring" (digestion of the hemoglobin by the parasite). "There is, therefore, in this form a very diaphanous portion of the parasitic body which prevents a perfect appreciation of the color of the corpuscle. . . . The blue ring is the protoplasm of the parasite which includes a nutritive vacuole."

After a parasite has completely decolorized, or dehemoglobinized, a red corpuscle, it usually assumes a compact form,—the ring shape is lost and either a very small attaching area is seen or the pseudopodia are entirely withdrawn by the parasite. I believe this to be a stage preparatory to migration. Bass and Johns<sup>3</sup> possibly refer to a similar appearance: "When parasites die . . . the central clear space in the small ring closes, or if the parasite is older, the irregular projections, pseudopodia, are withdrawn."

*The Parasitic Achromatic Area.*—This area closely surrounds the chromatin substance in the young ring-form parasite (plates 56 to 58), and in some instances shows quite distinctly in contrast with the deeply colored corpuscular substance (plate 57, figure 4; plate 59, figure 3). The parasitic achromatic area increases in size with the growth of the parasite (plate 60, figures 5 to 12, and 14 and 15). This area is not always achromatic (plate 60, figure 11), but may stain either a delicate blue (plate 60, figure 15), or a pink (plate 60, figures 9 and 10).

When this area surrounding the chromatin substance of the parasite is unstained, and overlies the area of corpuscular substance which has been decolorized by the action of the parasite surrounding it, it may be impossible to differentiate the one colorless area from the other; the chromatin mass may thus appear to be separated from the blue-stained protoplasm of the parasite and to lie within and belong to the colorless corpuscular area, thus giving rise, probably, to the erroneous belief that the colorless corpuscular area is a part of the parasite itself (plate 59, figures 25 and 26). But even in these instances a careful scrutiny of the parasite will often show a slight differentiation between the colorless corpuscular area and

<sup>3</sup> Bass, C. C., and Johns, F. M., *Jour. Exper. Med.*, 1912, xvi, 567.

the achromatic area belonging to the parasite (plate 58, figures 8, 12, and 13).

The latter area may stand out clear and sharp against the unstained background of the blood smear when the chromatin mass of the parasite extends beyond the periphery of the red corpuscle to which the parasite is attached (plate 56, figures 12, 13, and 34; plate 58, figure 7, A), or when the parasite is free (plate 60, figures 12 and 17) in the blood serum.

That this achromatic area which closely surrounds the chromatin mass of the parasite, and which is probably a part of the nucleus, is sometimes opaque or not entirely transparent is shown by the fact that this area can be seen when the infected corpuscles overlies one another.

*Infection of a Corpuscular Mound by Two or More Parasites.*—Infection of a corpuscular mound by more than one parasite (plate 58, figures 11, and 14 to 34; plate 59, figures 7, 8, 20, 25, and 26) may be seen in all the malarial infections. The best examples are observed in the æstivo-autumnal infections. As might be expected, instances of multiple infection are most frequently met with in cases where the parasites are numerous.

The majority of young parasites occupying the one corpuscular mound are in similar stages of development (plate 58, figures 15 to 21, 23, 25, 27, and 30 to 33; plate 59, figures 7, 8, 20, 25, and 26). This similarity in size is easily explained, as the parasites are probably of one brood, and it may be the result of one segmenting parasite. Parasites of varying sizes occupying the one corpuscular mound (plate 58, figures 11 and 22) may be explained by supposing that a young parasite attaches itself to a corpuscular mound already occupied by a parasite of an older brood.

The parasites seem to follow no rule as to their position in relation to each other in the occupancy of the one corpuscular mound (plate 58). And I have found no evidence of conjugation. The bodies or nuclei do not coalesce, as with careful examination with an apochromatic lens of high power the parasites can be differentiated, one from the other, even in some of the cases where the parasites are actually superimposed (plate 58, figures 21, 29, 30, and 31).



In cases of multiple infection of a corpuscular mound, the parasites seem to proceed with the destruction of the mound in the same way that the single parasites do. Occasionally one of the young parasites may show a grain of pigment in its protoplasm (plate 58, figure 25).

Multiple infection of a corpuscular mound should not be confused with the appearance produced by a single parasite whose protoplasm is intact, but whose nucleus is broken up by the technique used in spreading the smears. Such parasites with nuclei forcibly separated into parts, which may be of varying sizes, are usually found along the edges of smears made on slides, and the interpretation is made easy by the fact that parasites with nuclei similarly distorted may be found in groups. A young parasite never has more than one nucleus.

*The Supposed Conjugation of Parasites.*—The close approximation of two parasites occupying the same corpuscular mound has undoubtedly led to many conceptions or theories as to the conjugation of malarial parasites; but the fact that the corpuscular mounds occupied by more than one parasite may be seen at the periphery of the infected red corpuscle (plate 58, figures 11, 14, and 26 to 29) makes my interpretation less difficult than it might otherwise be.

Mannaberg (41), in 1894, described what he believed to be a process of conjugation; in the first he shows the young ring-form parasites coalesce with each other to form larger bodies, these latter later developing into crescents. He pictured, as an early stage in the process, two young ring-form parasites side by side, usually at the periphery of the red corpuscle. "The outer contour of both parasites frequently coalesces without interruption, and falls together with the edge of the blood corpuscle; the inner contour is often of a concave shape." This description, as well as Mannaberg's illustration of the same, corresponds with that of two young ring-form parasites attached to adjoining corpuscular mounds on the one red corpuscle at its periphery (plate 58, figure 8). Two young ring-forms, attached to the same corpuscular mound, with the bodies of the parasites at opposite poles, may resemble a small crescent (plate 58, figure 33).

Ewing, in 1901, described a process of conjugation between the parasites of tertian infections. He was led to believe that the parasites conjugated, because in certain smears of malarial blood studied by him nearly all the younger parasites were twinned, while all the older forms were single. These facts are explained by the migration of the younger malarial parasites to other corpuscles. He describes the process of conjugation as beginning with two young parasites

of varying morphology, depicting first a union of the protoplasm of the young parasites, the fusion of the nuclei taking place at a later stage in the development of the conjugating pair. In illustration of the first stage he pictures two young parasites, side by side, each attached to its own corpuscular mound, with their peripheries touching or overlying. A later stage illustrates adult parasites with two or three groups of chromatin granules "surrounded by one achromatic zone."

This last stage represents, I believe, the fastening of two or three adult parasites, by the pseudopodia arising near their nuclei, to the one corpuscular mound, which has become decolorized by the action of the parasites (plate 59, figures 25 and 26). The groups of chromatin granules are separated from each other and from the parasitic body by the achromatic areas belonging to the parasites.

The appearance of fusion between the protoplasm of two adult parasites is seen where two accessory pseudopodia encircle the same corpuscular mound, one being superimposed over the other.

In 1905 Craig (43) described a process of "intracorpuseular conjugation" of young ring-form malarial parasites. He figures three stages in the conjugation process: in the first, he shows the young ring-form parasites, side by side, their protoplasm touching or overlapping at the periphery; in the second stage, besides the parasites described in the first stage, he pictures what appears to be two young parasites occupying the same corpuscular mound; and in the third stage he shows (a) parasites attached to the corpuscle on separate mounds but in close proximity, (b) what appear to be parasites with distorted nuclei, (c) multiple infection of corpuscular mounds, and (d) large ring-form parasites with single nuclei. He interprets the last as resulting from conjugation.

Craig's description of the morphology of these parasites corresponds with that of the two parasites occupying one corpuscular mound, with the exception, perhaps, that he has never observed the presence of pigment in the protoplasm of these parasites. In discussing his disagreement with Ewing's theory, he states that he does not see any difference in the morphology of the conjugating parasites, for "while, in rare instances, one of the organisms may appear *slightly larger than the other*, may have a larger mass of chromatin, and *stain more intensely*, in the vast majority of instances the two conjugating parasites are of exactly the same size, and cannot be distinguished one from the other in any way."

I have not been able to find any evidence of conjugation of malarial parasites in any stage of development, and it may be that Craig's "conjugating parasites" are the result of a wrong interpretation of the infection of a corpuscular mound by two young parasites. The idea that the parasites were intracellular perhaps led to the misconception.

FREE PARASITES WITH ATTACHING PSEUDOPODIA IN THE FORM  
OF LOOPS.

The majority of free parasites seen in malarial infections have a compact structure. Rarely free parasites are seen with pseudopodia extended for attachment (plate 56, figures 2 to 5; plate 59, figures 1 and 6), and occasionally free parasites are seen with their pseudopodia in the form of loops, with or without the nuclei at the extremity of the loops (plate 60, figures 16 to 22). These free parasites with the loop-form pseudopodia are in migration, and have been set free while their pseudopodia are still in the form assumed by the parasites for attachment to corpuscular mounds (plate 60, figure 23).

I wish to thank Dr. William T. Councilman and Dr. Richard C. Cabot, of Harvard University, for their advice and encouragement during the prosecution of the research; and to acknowledge my appreciation and indebtedness to Dr. William H. Welch and Dr. William S. Thayer, of Johns Hopkins University, for their verification of my work after examination of the smears.

BIBLIOGRAPHY.

1. Laveran, A., *Paludism*, translated by Moore, J. W., London, 1893, 11.
2. Richard, *Compt. rend. Acad. d. sc.*, 1882, xciv, 496.
3. Richard, E., *Rev. scient.*, 1883, 114.
4. Mannaberg, J., *Malarial Parasites*, London, 1894, 276-277, plate II, figures 41 and 42.
5. Marchiafava, J., and Bignami, A., in *Twentieth Century Practice of Medicine*, New York, 1900, xix, 59, plate IV, figure 24.
6. Ewing, J., *Jour. Exper. Med.*, 1900-1, v, 475.
7. Thayer, W. S., and Hewetson, J., *Johns Hopkins Hosp. Rep.*, 1895, v, 91.
8. Craig, C. F., in Osler, W., and McCrae, T., *A System of Medicine*, London, 1907, i, 408.
9. Thayer, W. S., and Hewetson, J., *loc. cit.*, p. 79.
10. Thayer, W. S., *Lectures on the Malarial Fevers*, New York, 1897, 43.
11. Da Costa, J., *Clinical Hæmatology*, Philadelphia, 1901, 43.
12. Ewing, J., *Clinical Pathology of the Blood*, 2d edition, New York and Philadelphia, 1903, 436.
13. Osler, Councilman, and Antolisei, cited by Marchiafava, J., and Bignami, A., *loc. cit.*, p. 33.
14. Marchiafava, J., and Bignami, A., *loc. cit.*
15. Mannaberg, J., *loc. cit.*, p. 349.
16. Marchiafava and Celli, cited by Marchiafava, J., and Bignami, A., *loc. cit.*, p. 32.
17. Celli, A., and Guarnieri, G., *Fortschr. d. Med.*, 1889, vii, 521.

18. Mannaberg, J., *loc. cit.*, p. 349.
19. Thayer, W. S., and Hewetson, J., *loc. cit.*, p. 47.
20. Bastienelli and Bignami, cited by Thayer, W. S., and Hewetson, J., *loc. cit.*, p. 47.
21. Ziemann, H., *Über Malaria- und andere Blutparasiten nebst Anhang eine wirksame Methode der Chromatin- und Blutfärbung*, Jena, 1898.
22. Opie, E. L., in *Twentieth Century Practice of Medicine*, New York, 1900, xix, 788.
23. Rowley-Lawson, M., *Jour. Exper. Med.*, 1911, xiii, 263.
24. Argutinsky, cited by Calkins, G. N., *Protozoölogy*, Philadelphia and New York, 1909, 283.
25. Nocht, cited by Calkins, G. N., *loc. cit.*, p. 283.
26. Sakharoff, N., *Ann. de l'Inst. Pasteur*, 1891, v, 445.
27. Canalis, cited by Thayer, W. S., and Hewetson, J., *loc. cit.*, p. 22.
28. Celli and Sanfelice, cited by Thayer, W. S., and Hewetson, J., *loc. cit.*, p. 46.
29. Grassi and Feletti, cited by Thayer, W. S., and Hewetson, J., *loc. cit.*, p. 45.
30. Romanovski, D. L., *Vrach*, 1890, xi, 1171.
31. Manson, P., *Tropical Diseases*, 1900, 8.
32. Osler, W., *The Principles and Practice of Medicine*, 5th edition, New York, 1904, 205.
33. Cabot, R. C., *Malaria, Clinical Examination of the Blood*, 4th edition, New York, 1904, 465.
34. Craig, C. F., *Arch. Int. Med.*, 1910, v, 325.
35. Marchiafava, J., and Bignami, A., *loc. cit.*, p. 34.
36. Schaudinn, cited by Calkins, G. N., *loc. cit.*, p. 283.
37. Maurer, cited by Loeffler, F., *Modern Clinical Medicine, Infectious Diseases*, New York, 1910, 255.
38. Calkins, G. N., *loc. cit.*, p. 283.
39. Loeffler, F., *loc. cit.*, p. 216.
40. Marchiafava, J., and Bignami, A., *loc. cit.*, p. 34.
41. Mannaberg, J., *loc. cit.*, pp. 289-294.
42. Ewing, J., *Jour. Exper. Med.*, *loc. cit.*
43. Craig, C. F., *Am. Med.*, 1905, x, 983-984.

## EXPLANATION OF PLATES.

THE EXTRACELLULAR RELATION OF THE MALARIAL PARASITE TO THE RED CORPUSCLE.  
THE METHOD BY WHICH THE PARASITE SECURES ITS ATTACHMENT TO  
THE SURFACE OF THE RED CORPUSCLE.

### PLATE 56.

#### ÆSTIVO-AUTUMNAL PARASITES.

Magnification,  $\times 1,900$ .

FIG. 1. The youngest form of the æstivo-autumnal parasite, resulting from a recent segmentation. This figure is used as a standard of comparison as to the relative sizes of the parasites illustrated.

FIGS. 2 to 4. Young parasites free in the blood serum, showing filamentous pseudopodia arising from the cytoplasm of the parasites. Fig. 2 shows well the origin of the pseudopodia near the nucleus of the parasite.

FIG. 5. A young parasite, free in the blood serum, showing the attaching pseudopodium in the form of a loop.

FIGS. 6 to 11. Early stages in the attachment of the parasite to the red corpuscle by means of the pseudopodium arranged in a loop, overlying the corpuscular substance. The parasitic body is on the periphery of the red corpuscle.

FIG. 12. A young parasite on the periphery of the red corpuscle, starting to pull up the corpuscular mound.

FIGS. 13 to 35. Parasites in various positions attached to the surface of the red corpuscle by means of their delicate, thread-like pseudopodia encircling mounds of corpuscular substance at the periphery of the red corpuscle. The mound of corpuscular substance has been pulled up by the parasite apparently for the purpose of attachment and digestion. Note that the surrounding filament can be seen in its entirety in many of the figures (i. e., Figs. 18, 19, 20, and 29). In many of the parasites an achromatic area can be seen closely surrounding the chromatin granule of the parasite. Figs. 26, 33, and 35 show minute granules of pigment in the cytoplasm of the parasites. Fig. 22 shows the parasite overlying the corpuscular mound. The encircling filament can be seen at o.

#### PLATE 57.

##### ÆSTIVO-AUTUMNAL PARASITES.

Magnification,  $\times 1,900$ .

FIGS. 1 to 10. Parasites attached to the external surface of the red corpuscle by means of their pseudopodia which encircle the corpuscular mounds at the periphery of the red corpuscles, the mounds having been pulled up by the parasites for the purpose of attachment. The pseudopodium of the upper parasite in Fig. 2 can be seen encircling the peripheral mound. In Fig. 7 the parasite can be seen on both surfaces of the corpuscle. The part of the parasite on the under surface can be seen at o.

FIGS. 11 and 12. These figures show, I believe, what has been described as "retraction of hemoglobin"; i. e., clear unstained areas at the periphery of the corpuscles. This condition is due to the fact that the parasite, in attaching itself to the corpuscle, has pulled up the hemoglobin in such a manner as to leave decolorized areas at the periphery of the cell. The condition is not the result of destruction of the hemoglobin substance. At o in Fig. 12, two parasites can be seen surrounding the mound of hemoglobin which has been pulled up from the clear areas at A.

FIG. 13. A parasite attached to a peripheral corpuscular mound.

FIG. 14. A portion of the body of the attached parasite on the under surface of the corpuscle at oo.

FIG. 15. The protoplasm of the attached parasite conforming to the shape of the corpuscular mound is seen.

FIG. 16. A parasite attached to a hemoglobin mound near the periphery of the corpuscle. The pseudopodium of the parasite can be seen encircling the mound at its base.

FIGS. 17 to 23. Parasites attached to peripheral corpuscular mounds which are in various stages of dehemoglobinization (pallor) due to the gradual digestion of the hemoglobin by the parasites.

FIG. 21. Crenation of the corpuscular mound. Fig. 22 shows the parasitic body on both surfaces of the corpuscle.

FIGS. 24 and 25. Parasites attached to peripheral decolorized corpuscular mounds which have been completely dehemoglobinized by the action of the parasites. The decolorization does not extend below the encircling pseudopodia.

FIGS. 26 to 32. Parasites attached to the upper surface of the red corpuscles by means of pseudopodia surrounding corpuscular mounds. Figs. 26, and 29 to 32 show the mounds pressed down over the surface of the red corpuscles. Fig. 32 shows the pseudopodium of the parasite encircling the mound.

FIG. 33. Here the parasite appears to have pulled up the entire lower periphery of the red corpuscle into the mound to which it is attached.

FIGS. 34 and 35. Parasites attached to corpuscular mounds, one on the surface, the other at the periphery of the red corpuscle. The nuclei of these parasites conform to the contour of the mounds.

## PLATE 58.

### ÆSTIVO-AUTUMNAL PARASITES.

Magnification,  $\times 1,900$ .

FIGS. 1 to 6. Parasites attached to corpuscular mounds. Fig. 3 shows the mounds at the periphery of the corpuscle; the other figures show the mounds on the surface of the corpuscles. When the parasites are attached to the red corpuscles in the latter position, with their pseudopodia surrounding the corpuscular mounds, the characteristic ring-form picture is seen.

FIG. 7. Two parasites attached to corpuscular mounds on the surface of the red corpuscle. The parasite at A has recently attached itself and the hemoglobin of the mound is still unaltered. At o the parasite is seen encircling a completely decolorized mound, with an accessory filament attached to another mound at x.

FIG 8. Two parasites surrounding with their pseudopodia completely decolorized corpuscular areas. This figure corresponds to a stage in the formation of crescents from the fusion of two young ring-form parasites, as described and pictured by Mannaberg.

FIG. 9. A parasite attached to a corpuscular mound on the upper, external surface of the red corpuscle. A partial decolorization of the corpuscular mound can be seen.

FIG. 10. Complete decolorization, or dehemoglobinization, of the corpuscular area, which is encircled by the pseudopodium of the parasite.

FIG. 11. A peripheral corpuscular mound, encircled by two young parasites of varying sizes.

FIGS. 12 and 13. Parasites surrounding decolorized, or dehemoglobinized, corpuscular mounds. Here the nuclei conform to the contour of the mounds. These figures illustrate how much damage can be done to a corpuscle by a very young parasite, without any evidence of the presence of pigment in the protoplasm. That these corpuscular mounds are transparent, is shown by the fact that in Fig. 12 the periphery of the adjoining uninfected red corpuscle can be seen through the achromatic area, which overlies it; and in Fig. 13, the parasite underneath can be seen through the achromatic area of the overlying corpuscle.

These figures correspond with Mannaberg's and Marchiafava and Bignami's description and drawings of parasites resting in niches at the periphery of the red corpuscles.

FIG. 14. Two parasites attached to one peripheral corpuscular mound. A is on the under surface of the corpuscle, o on the upper surface. Note the difference in clearness and sharpness of outline between the two parasites.

FIG. 15. Two parasites, in similar stages of development, encircling a corpuscular mound at the periphery of the red corpuscle. The pseudopodium of the parasite on the left can be seen encircling the mound at o.

FIGS. 16 to 18. The gradual destruction, or decolorization, of the corpuscular mound encircled by parasites at opposite poles is seen.

FIG. 19. Two parasites occupying the one decolorized corpuscular mound.

FIG. 20. Two parasites, with bodies at opposite poles, occupying the one corpuscular mound.

FIG. 21. Two parasites, with bodies superimposed, occupying the one corpuscular mound.

FIG. 22. Parasites of varying sizes occupying the same corpuscular mound.

FIG. 23. Two parasites, in similar stages of development, occupying one corpuscular mound. At o the body of the lower parasite can be seen overlying that of the upper parasite.

FIG. 24. Three parasites, in similar stages of development, occupying one corpuscular mound. A careful examination of the picture will enable one to trace the outline of each parasite.

FIG. 25. Two parasites on a corpuscular mound near the periphery of the corpuscle. The body of the parasite at o extends beyond the periphery of the corpuscle and shows a granule of pigment in the protoplasm.

FIG. 26. Two parasites attached to the same peripheral corpuscular mound. One of the parasites is attached to the upper, the other to the under surface of the corpuscle.

FIGS. 27 and 28. Two parasites attached to one peripheral corpuscular mound.

FIG. 29. Three parasites attached to one peripheral corpuscular mound. At o one of the parasites is superimposed over the other.

FIGS. 30 and 31. Two parasites, one superimposed over the other, attached to the one corpuscular mound.

FIG. 32. Two parasites attached to the one decolorized corpuscular mound.

FIG. 33. Two parasites attached to the one corpuscular mound in such a manner that they resemble in shape a small crescent.

FIG. 34. Three young parasites attached to the one corpuscular mound.

#### PLATE 59.

#### TERTIAN PARASITES.

Magnification,  $\times 1,900$ .

FIG. 1. Two young parasites, the result of a recent segmentation. The delicate attaching pseudopodium can be seen formed from the cytoplasm of the parasite and arising near the nucleus.

FIG. 2. A young parasite attached to a peripheral corpuscular mound.

FIG. 3. A young parasite attached to a hemoglobin mound on the upper surface of the corpuscle; the protoplasm of the parasite extends beyond the periphery of the corpuscle.

FIG. 4. A young parasite attached to a peripheral corpuscular mound.

FIG. 5. A young parasite attached to a decolorized peripheral corpuscular mound. There is no evidence of pigment in the protoplasm of the parasite.

FIG. 6. A free parasite, showing the pseudopodium but little extended.

FIG. 7. Two young parasites attached to one hemoglobin mound at the periphery of the red corpuscle. The nucleus of the upper parasite extends beyond the periphery of the corpuscle.

FIG. 8. Two parasites attached to a decolorized corpuscular mound.

FIG. 9. A ring-form parasite attached to the upper surface of the red corpuscle, the entire protoplasm of the parasite entering into the formation of the pseudopodium. The small achromatic area, which is parasitic, can be seen surrounding the chromatin substance.

FIG. 10. A parasite encircling a completely decolorized corpuscular area. A granule of pigment is seen in the protoplasm of the parasite.

FIG. 11. An adult, pigmented parasite attached to a peripheral corpuscular mound at the periphery of the red corpuscle at o. The pseudopodium of the parasite can be distinctly seen encircling the mound at its base.

FIG. 12. An adult, pigmented parasite attached to a peripheral corpuscular mound. Beginning decolorization of the corpuscles is seen at o.

FIGS. 13 to 17. Adult, pigmented parasites attached to corpuscular mounds at o. Fig. 15 shows a parasite with an accessory filament surrounding a secondary corpuscular mound at x.

FIG. 18. A parasite attached to a granular corpuscular skeleton, by means of its pseudopodium encircling a mound at o. The periphery of the corpuscular skeleton can be seen through the mound.

FIG. 19. Two young pigmented parasites attached to corpuscular mounds at o. The corpuscle shows Schuffner's granulation.

FIG. 20. Two adult, pigmented parasites attached to separate mounds, side by side, at oo.

FIGS. 21 to 24. Parasites attached to corpuscular mounds at o.

FIGS. 22, 23, and 24 show the mounds in various stages of destruction.

FIGS. 25 and 26. Two adult parasites attached to one corpuscular mound. These figures correspond to certain stages depicted by Ewing in his conjugation theory.

## PLATE 60.

### TERTIAN PARASITES.

Magnification,  $\times 1,900$ .

FIG. 1. A young parasite encircling with its pseudopodium a completely decolorized corpuscular mound. The infected corpuscle shows Schuffner's granulation. This is evidence that Schuffner's granulation forms a part of the substance which is digested by the parasite.

FIG. 2. A parasite encircling an almost completely decolorized corpuscular mound, the corpuscle showing Schuffner's granulation.

FIG. 3. A young parasite encircling with its pseudopodium an almost completely decolorized corpuscular mound. This corpuscle shows blue granular stippling.

FIG. 4. A young parasite encircling a completely decolorized corpuscular mound. This corpuscle also shows blue granular stippling. The evidence here



suggests that the blue stippling also forms a part of the substance digested by the parasite.

FIGS. 5, 6, and 7. Parasites surrounding corpuscular mounds on the upper surface of the red corpuscles. The area which belongs to the parasite and surrounds the nucleus is unstained and shows well against the hemoglobin of the corpuscles.

FIG. 8. An adult, pigmented parasite, whose pseudopodia can be seen arising from the cytoplasm of the parasite near its nucleus. They seem to end, one on either side of the decolorized corpuscular mound at the periphery of the corpuscle at o.

FIGS. 9 and 10. The parasitic area surrounding the chromatin substance is stained pink, and contains no pigment granules.

FIG. 11. The parasitic area surrounding the chromatin granules is unstained. When this area is unstained and overlies a mound of corpuscular substance that has been completely decolorized by the action of the parasite, it may be impossible to differentiate one achromatic area from the other, thus giving the chromatin granules the false appearance of being entirely separated from the blue stained protoplasm of the parasite, and of belonging to the decolorized corpuscular area.

FIG. 12. A free, pigmented parasite, showing the achromatic area surrounding the chromatin granules.

FIGS. 13 and 14. Two adult, pigmented parasites, on the periphery of the red corpuscles, the corpuscles showing evidence of degenerative changes due to the action of the parasites.

FIG. 15. An adult, pigmented parasite, attached to the upper surface of the red corpuscle. The nucleus of the parasite extends beyond the periphery of the infected corpuscle and overlies the adjoining red corpuscle at o.

FIGS. 16 to 22. Free parasites with their pseudopodia in the form of loops, with the nuclei, with the exception of Fig. 16, at the extremity of the loop. These parasites have destroyed the corpuscles to which they were attached and are in migration, with the pseudopodia still in the form of the loops which had encircled the corpuscular mounds.

FIG. 23. A parasite attached to a corpuscular mound by a loop similar to those seen in connection with Figs. 17 to 22.

#### PLATE 61.

FIG. 1. A free æstivo-autumnal parasite with loop attachment at o; also an æstivo-autumnal parasite with body and nucleus on the periphery of the red corpuscle. The pseudopodium is in the form of a loop, encircling a mound of unaltered hemoglobin which the parasite has pulled up for the purpose of attaching itself to it.

FIGS. 2 to 4. The gradual decolorization, or dehemoglobinization, of that portion of the corpuscle which lies within the boundary of the encircling pseudopodium of the parasite. This condition results from the gradual digestion of the hemoglobin substance by the parasite.

FIGS. 5 to 8. Æstivo-autumnal parasites attached in various positions to the surface of the red corpuscles by means of pseudopodia encircling hemoglobin

mounds at the periphery of the red corpuscles. These mounds have been pulled up by the parasites for the purpose of attachment and digestion.

FIG. 9. An æstivo-autumnal parasite attached to a hemoglobin mound at the periphery of the red corpuscle. This parasite can be seen on both surfaces of the red corpuscle; the part on the under surface can be seen at o.

FIG. 10. An æstivo-autumnal parasite attached to a corpuscular mound which has been completely decolorized, or dehemoglobinized, by the action of the parasite.

FIG. 11. Two æstivo-autumnal parasites attached to the same hemoglobin mound at the periphery of the red corpuscle, their pseudopodia encircling the mound at its base. The parasite on the right is on the upper surface of the corpuscle; the parasite on the left is on the under surface and, being seen through the substance of the corpuscle, shows less distinctly than the one on the right.

FIG. 12. An æstivo-autumnal parasite attached to the upper surface of the red corpuscle by means of its pseudopodium encircling a hemoglobin mound, which has been pulled up by the parasite. The mound can be seen to be pressed down on the surface of the corpuscle.

FIGS. 13 and 14. Two æstivo-autumnal parasites attached to the upper surface of the red corpuscle by means of pseudopodia encircling one hemoglobin mound. These two figures correspond to the parasites described by Craig as "conjugating young parasites."

FIG. 15. Two young æstivo-autumnal parasites attached, side by side, to separate decolorized corpuscular mounds. These parasites correspond to the parasites described by Mannaberg as "paired parasites," which he believed coalesced to form a crescent.

FIG. 16. Two young æstivo-autumnal parasites attached to one decolorized corpuscular mound, resembling, in this relation to each other, a small crescent.

FIG. 17. Two young tertian parasites, one slightly thicker than the other, attached side by side to separate hemoglobin mounds, the periphery of one ring overlying that of the other ring. These parasites correspond to the parasites described by Ewing as "conjugating rings of unequal size."

FIG. 18. Two adult, pigmented, tertian parasites, attached to one decolorized corpuscular mound. A slight separation can be seen between the two groups of chromatin granules. The accessory pseudopodia of these parasites are superimposed while surrounding a secondary hemoglobin mound at o. These parasites correspond to the parasites described by Ewing as the result of "a complete union of bodies and nuclei."

FIG. 19. An adult, tertian parasite surrounding with its pseudopodium a decolorized corpuscular mound. The chromatin mass is at the extremity of the loop.

FIG. 20. A free tertian parasite, showing the pseudopodium in the form of a loop with the chromatin mass at the extremity of the loop.

FIG. 21. A tertian parasite showing the pink stained area surrounding the chromatin granules. The red corpuscle shows Schuffner's granulation.

FIG. 22. A tertian parasite, showing the blue stained area surrounding the chromatin granules. The corpuscular mounds to which the parasite is attached are decolorized by the action of the parasite.

FIG. 23. A tertian parasite with an unstained area surrounding the chromatin granules. The red corpuscle shows Schuffner's granulation.

FIG. 24. A tertian parasite with an unstained area which is a part of the parasite, and which surrounds the chromatin granules, and overlies a mound of corpuscular substance that has been completely decolorized by the action of the parasite. It may be impossible to differentiate one achromatic area from the other. This appearance might lead an observer to believe the entire decolorized area to be parasitic.



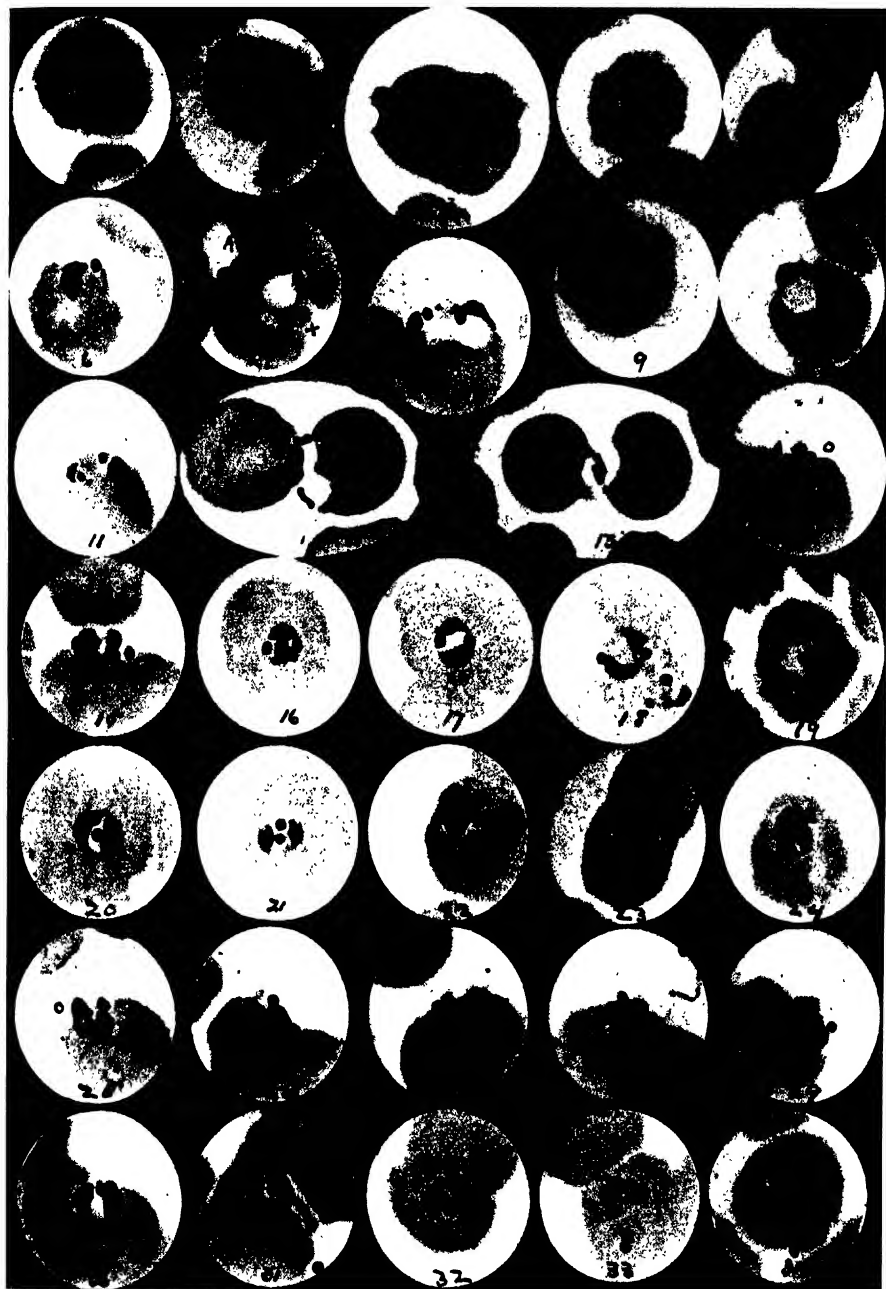
(Lawson : Relation of Malarial Parasite.)





(Lawson : Relation of Malarial Parasite.)





(Lawson : Relation of Malarial Parasite.)

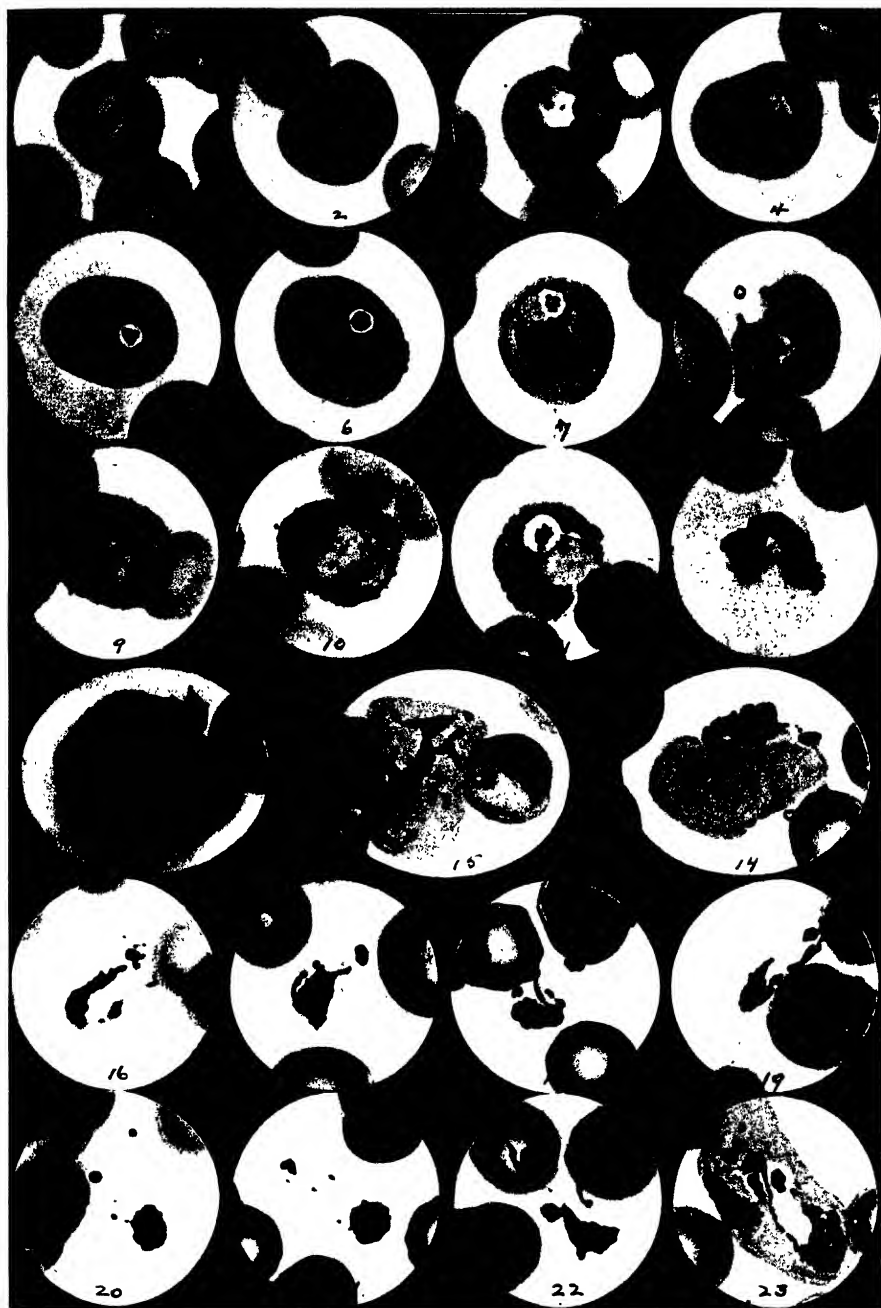






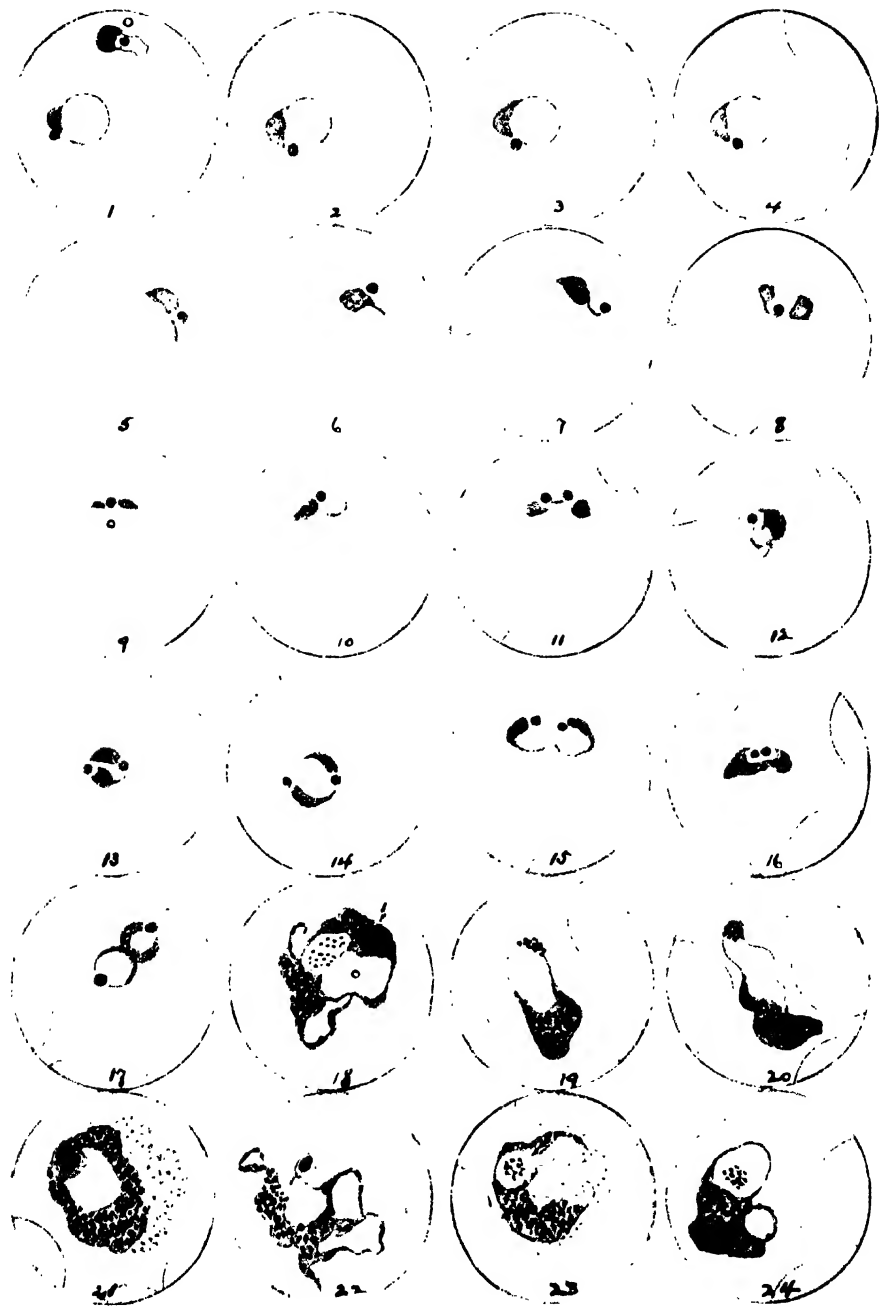
(Lawson: Relation of Malarial Parasite.)





(Lawson : Relation of Malarial Parasite.)





(Lawson : Relation of Malarial Parasite.)



## ON THE RESISTANCE OF VARIOUS SPIROCHAETES IN CULTURES TO THE ACTION OF CHEMICAL AND PHYSICAL AGENTS.\*

By J. BRONFENBRENNER AND H. NOGUCHI.

*(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)*

The resistance of various microörganisms to the action of different chemical compounds and temperature had been thoroughly studied by many investigators, but that of spirochaetes remained so far undetermined, due to the lack of cultural methods. Recently one of us succeeded in isolating certain pathogenic and non-pathogenic varieties of the latter class of organisms in pure cultures and made it possible to find out how they would behave towards the action of some of the most commonly employed chemicals and therapeutic compounds. As our main interest is centered around the organism of syphilis we have chosen bichloride of mercury and Salvarsan as the principal substances for the present series of investigations.

The cultures used in these experiments were isolated by Noguchi, while the experiments of determination of the resistance of these organisms were made by Bronfenbrenner.

The cultures used for these experiments were grown on solid media as described by Noguchi and taken in a capillary pipette and broken up in a sterile flask with glass beads in the presence of 0.9 per cent sodium chloride solution, and filtered through a sterile filter paper free from agar.

One-tenth cubic centimeter of this filtrate is put in a small Petri-dish and 0.5 cc. of different chemicals in dilutions as indicated below is added so that the final dilutions are in fact by one-sixth higher than indicated in the tables. After a contact at room temperature

\* Received for publication, February 1, 1913.



TABLE I.

	PALLIDUM (heavy type)		PALLIDUM (small type)		REFRIGENS		MUCOSUM		MICRODENTUM		BACTERIUM COLI	
	+	-	+	-	+	-	+	-	+	-	Inhibi- tion	Sterili- zation
HgCl <sub>2</sub> .....	300,000	200,000	700,000	500,000	300,000	200,000	400,000	300,000	400,000	300,000	16,000	16,000
AsO <sub>3</sub> .....	40,000	30,000	30,000	20,000	5,000	3,000	40,000	30,000	50,000	40,000	2,000	500
Triresol.....	750	500	750	500	750	500	2,000	1,000	750	500		
Phenol.....	400	200	400	200	400	200	400	200	300	200		
Saponin.....	7,500	5,000	10,000	7,000	10,000	5,000	5,000	3,000	5,000	3,000	100	100
Sodium tauro- cholate.....	2,000	1,000	3,000	2,000	3,000	2,000	5,000	4,000	10,000	5,000	100	100
NaOH.....	N	N	N	N	N	N	N	N	N	N		
HCl.....	N	N	N	N	N	N	N	N	N	N		
Gentian violet ..	500	300	500	300	500	200	500	200	500	200		
Alcohol.....	>10		>10		>10		>10		>10			
Old "606".....	2,000	1,000	1,500	1,000	1,500	1,000	2,000	1,000	3,000	2,000		

for one hour the contents of the dishes are transferred with the sterile capillary pipette into tubes, and the constituents of culture media subsequently added.

On Table I the numbers in the columns under the plus sign indicate the dilutions of chemicals at which the organisms grew, and those under the minus sign indicate the highest dilutions which exerted sterilizing action.

The results of these experiments were taken according to a microscopical examination with the dark-field illumination after ten days incubation at 37°C.

In the case of *Bacterium coli* the concentrated chemicals were diluted by means of bouillon and after inoculation with two loops of uniform suspension of *Bacterium coli* incubated for twenty-four hours at the temperature of 37°C. The absence of turbidity in the culture tubes at the end of twenty-four hours was taken as a sign of inhibition of growth, and subsequent agar cultures were made to determine the actual sterilization. It is, therefore, under the heading of *Colon bacillus* on Table I that two columns are found, one of which indicates the dilution at which inhibition only occurs, and the other the complete sterilization as tested by subsequent agar cultivation.

The results tabulated above show the comparative sensitiveness of spirochaetes in general as being at least about twenty, and in a few instances even as much as one-hundred times greater than in the case of *Colon bacillus* tested with the same chemicals.

The alcohol was not tested especially but as a control for gentian violet which contained alcohol, and as it is shown even in as strong dilution as 1 : 10 alcohol does not sterilize spirochaetes.

As to the Salvarsan (or old "606") its sterilizing property is about equivalent to the same of the Neo-Salvarsan (see first column on Table III).

It was found that very minute quantities far below the sterilizing limits of antiseptics (phenol, trikresol, bichloride of mercury and arsenic compounds) exerted very marked stimulating action upon the growth of *Spirochaetes*.

In order to see whether the presence of protein in the ascitic fluid used for cultivation might have inhibited the sterilizing action of the chemicals the following experiment was performed :

A fixed amount of bichloride of mercury, representing several toxic doses and different amounts of ascitic fluid were added to the fixed amount of spirochaete suspension, and the volume was made uniform by means of a physiological solution. This mixture was allowed to stand at room temperature for one hour and then ascitic fluid, physiological salt solution and agar were added in the quantities indicated on Table II. The tubes are then incubated at 37° C. and the results of the experiment are taken after ten days.

TABLE II.

	CONTROLS						
	cc.	cc.	cc.	cc.	cc.	cc.	cc.
HgCl <sub>2</sub> 1:1000.....	0	0	0.5	0.5	0.5	0.5	0.5
Ascitic fluid.....	0	2	0	0.5	1	2	3
Salt solution.....	2.5	0	2.5	1.5	1	0	0
Microdentium.....	0.1	0.1	0.1	0.1	0.1	0.1	0.1
<i>One Hour at Room Temperature.</i>							
Ascitic fluid.....	3	1	3	2.5	9	1	0
Salt solution.....	0	2	0	0.5	1	2	2
<i>Rabbit's Tissue and 10 Cc. of Ascitic Agar in Each Tube.</i>							
Results after ten days.....	+	+	-	-	-	-	-

Thus it was found that the addition of ascitic fluid in the conditions of experiment does not interfere with the action of the chemical used.

In the experiments tabulated above we determined the sterilizing activity of Salvarsan by simply mixing it with the spirochaetes.

In order to see what effect upon spirochaetes Salvarsan may exert in the body we tried to change the plan of our experiments so as to approach its action in the living organism. For this purpose 0.1 of a gram of Neo-Salvarsan was dissolved in 10 cc. of distilled water (resulting in a dilution 1:100). Of this dilution 0.1 cc. was put into a series of tubes and 0.5, 0.7, 1 cc., 2 cc., etc., until 10 cc. of different diluents, as indicated on Table III, were added, resulting in dilutions of Salvarsan-1:500, 1:700, 1:1000, 1:2000, etc., until 1:10,000 respectively. The diluents used were: Physiological salt solution, sterile liver extract, same extract boiled for fifteen minutes, and rabbit's defibrinated blood (whole). The

tubes were then incubated for three hours at a temperature of 37°C.

At the end of incubation 0.5 cc. from each of the tubes was mixed in a Petri-dish with 0.1 cc. of cultures of spirochaetes on liquid media, and left at room temperature for one hour. After this the contents of the Petri-dishes were transferred, by means of sterile pipettes into tubes for cultivation, and 10 cc. of nutritive media and a piece of sterile rabbit tissue were added to each tube.

Two different strains of spirochaetes were used in this experiment, one a strain of pallidum belonging to heavy type, and the other a culture of spirochaeta refringens. The plus signs on the table indicate the dilutions in which at the end of ten days growth could be detected by means of a microscopic examination under the dark-field. The minus signs indicate absence of growth.

To see if the amount of Neo-Salvarsan in the tubes interfered with the growth a control tube was made by mixing, without previous incubation 0.1 cc. of the same spirochaete suspension with 0.5 cc. of Neo-Salvarsan in dilution 1 : 1000 in physiological salt solution, and as the table indicates, this amount of Neo-Salvarsan did not prevent the growth of spirochaete.

TABLE III.

DILUTION OF NEOSALVARSAN IN DIFFERENT DILUENTS	PHYSIOLOGICAL SOLUTION		LIVER EXTRACT		BOILED LIVER EXTRACT		RABBIT'S BLOOD		CONTROLS	
	Refringens	Pallidum	Refringens	Pallidum	Refringens	Pallidum	Refringens	Pallidum	Refringens	Pallidum
1 : 700.....	-	-	-	-	-	-	-	-		
1 : 1000.....	-	-	-	-	+	+	-	-	+	+
1 : 2000.....	+	< +	-	-	++	++	-	-		
1 : 3000.....	++	++	+	< +			-	-		
1 : 5000.....			+	+			< +	-		
1 : 7000.....			++	++			+	< +		
1 : 10000.....							+	+		

The first column of the Table III is comparable to the corresponding one on the Table I where Salvarsan (old) was simply diluted with physiological salt solution and mixed with spirochaetes. The columns 2, 3 and 4, however, give the idea of what may be taking place in the living organism. We see, especially from the column 4, that the presence of rabbit's blood increased the steriliz-

ing capacity of Neo-Salvarsan about five times and the liver extract at least increased it twice. We see also that the same extract being boiled—not only loses its ability to increase the toxic effect produced by Neo-Salvarsan upon spirochaetes, but on the contrary lessens it. This fact suggests once more the validity of the hypothesis that Neo-Salvarsan is broken up in the living organism, and that its derivatives are especially toxic for spirochaetes. The substances in the body that are capable of splitting Neo-Salvarsan may possibly be of enzyme nature because their activity is destroyed by boiling.

In order to study the effect of high temperatures upon spirochaetes the cultures were washed in physiological salt solution as above and 0.2 cc. of the resulting suspension, after filtration, was put into tubes and incubated in the water bath for different lengths of time at different temperatures as indicated on Table IV. After the incubation period in each case was completed a piece of sterile rabbit's tissue and ascitic agar were added to each tube and incubated at 37°C. for ten days, at the end of which period the contents of the tubes were examined by means of dark-field illumination. The plus signs on the table indicate the presence of growth, the minus signs indicate the absence of growth, or sterilizing action of the corresponding temperature.

TABLE IV.

	ROOM TEMPERATURE				37° C.			40° C.		45° C.				
	0	2 h.	6 h.	12 h.	2 h.	3 h.	6 h.	30 m.	1 h.	3 m.	5 m.	7 m.	10 m.	
Microdentium..	+	+	^	+	+	+	^	+	+	+	^	+	+	
Refringens.....	+	^	^	+	+	+	+	+	+	+	^	+	+	
Pallidum.....	+	+	+	^	+	+	^	+	+	+	+	+	+	
Pallidum heavy type.....	+	+	+	+	+	+	^	+	+	+	^	+	+	
Mucosum small type.....	+	+	+	+	+	+	^	+	+	+	^	+	+	

Although in a culture spirochaetes remain alive at room temperature for many weeks, yet as it results from above table at the end of twelve hours most of them are already dead. The reason for this is not the temperature, but the unfavorable conditions of experi-

ment as: lack of nutritive substances, presence of oxygen, effect of light, the toxic effect of sodium chloride, etc., all of which markedly diminish the viability of spirochaetes. As the temperature is heightened the viability of spirochaetes suffers more, as we would naturally expect and even at the end of six hours at 37°C. we already see marked differences between the tubes exposed respectively to room and 37°C. temperature. At the temperature of 45°C. the rate of this process increases so much that already at the end of seven minutes most, and at the end of ten minutes all of the organisms tested are dead.

On the other hand, the spirochaetes survive for many hours at 45°C. if we subject them to this temperature under the favorable conditions such as are provided in the growing culture tubes, viz., strictly anaerobic conditions, properly balanced saline constituents and other nutrient substances.

#### SUMMARY.

The toxic effect exerted by the chemicals in the experiments is from twenty to one-hundred times greater if tested upon spirochaetes than it is against *Colon bacillus*.

The toxic effects of Salvarsan are increased from two to five times and possibly more in the presence of enzymes from the liver and especially from the blood.

Spirochaetes suspended in physiological salt solution are sterilized by the temperature of 45°C. in from seven to ten minutes.

## THE RELATION OF THE LEUCOCYTIC BACTERIOLYSIN TO BODY FLUIDS.\*

By WILFRED H. MANWARING, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

The discovery that certain cells of the body are capable of giving off bactericidal substances has given rise to the hope that eventually cellular derivatives may prove of therapeutic value. It has been conceived, for example, that an efficient antiseptic may thus be obtained, which can be safely injected into body cavities and tissue spaces.

The bactericidal properties of many cellular products<sup>1</sup> have therefore been studied, particularly those of leucocytic products.<sup>2</sup> The leucocytic bacteriolysin has usually been tested, either dissolved in distilled water, in physiological saline, or in culture media. From observations in a previous paper,<sup>3</sup> I was led to test its properties when placed under conditions more nearly approaching those in the animal body. Its action was therefore tested when mixed with various normal and pathological body fluids and tissue derivatives.

The influence of a number of foreign substances on this bacteriolysin has already been studied. Of particular interest in the present connection is the observation of Pettersson,<sup>4</sup> that the bactericidal power is diminished in the presence of certain colloids. Pettersson<sup>5</sup> and others have shown that the leucocytic and the serum bacteriolysins are distinct substances. Kling and others, however, have observed an activation of an inactive leucocytic extract by the addi-

\* Received for publication, December 19, 1912.

<sup>1</sup> Conradi, H., *Beitr. z. chem. Phys. u. Path.*, 1902, i, 193.

<sup>2</sup> For a summary of previous work with leucocytic extracts, see Kling, C. A., *Ztschr. f. Immunitätsforsch.*, 1910, vii, 1. A review of the literature is also given by Schneider, R., *Arch. f. Hyg.*, 1909, lxx, 40.

<sup>3</sup> Manwaring, W. H., *Jour. Exper. Med.*, 1912, xvi, 249.

<sup>4</sup> Pettersson, A., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1911, lx, 286.

<sup>5</sup> Pettersson, A., *Ztschr. f. Immunitätsforsch.*, 1908-9, i, 52.

tion of serum. Weil, Schneider, and others have described the opposite effect, a diminution in bacteriolysis, as a result of serum addition.<sup>6</sup> Schattenfroh<sup>7</sup> found that the bactericidal power of an extract of rabbit leucocytes is independent of its salt content. Schneider<sup>8</sup> found that the addition of alkali diminishes the bactericidal power of the same extract.

#### MATERIAL AND TECHNIQUE.

The bacteriolysin studied in the present paper was obtained from horse leucocytes, according to the technique described in the previous communication. This extract had been heated to 57° C. for thirty minutes, had been dialyzed free from diffusible products, and then evaporated to dryness *in vacuo* at 45° C. It was tested after having been stored in a vacuum at 4° C. for about six months. The tests were made at room temperature.

#### EFFECTS OF SERUM ON THE BACTERIOLYSIN.

*Inactive Serum.*—The addition of a trace of inactive<sup>9</sup> homologous or foreign serum to an amount of leucocytic extract barely sufficient

TABLE I.

#### *Effect of Inactive Serum on the Bacteriolysin.*

The fluids to be tested were made up to equal volumes (1 c.c.) by the addition of  $\frac{1}{4}$  physiological saline solution (0.22 per cent. sodium chloride). Each fluid was then inoculated with a loopful of an eighteen hour broth culture of *B. typhosus*. Plates were made from the resulting mixtures at the times indicated. The table records the number of colonies on the plates thus obtained. The serum tested in this table was horse serum that had spontaneously become inactive by long standing in the ice chest.

Material tested.	Time of plating.				
	1 min.	1 hr.	2 hrs.	5 hrs.	24 hrs.
Bacteriolysin alone.....	950	90	10	8	650
Bacteriolysin + 0.5% inactive serum.....	510	7	0	0	15
50% inactive serum alone.....	1,000	960	1,010	1,220	16,000

<sup>6</sup> Kling, C. A., *loc. cit.*, p. 30.

<sup>7</sup> Schattenfroh, A., *Arch. f. Hyg.*, 1899, xxxv, 135.

<sup>8</sup> Schneider, R., *loc. cit.*, p. 120.

<sup>9</sup> Serum was usually inactivated, either by heating it to 57° C. for 30 min., or by allowing it to stand at room temperature till its bactericidal properties had disappeared.



for complete sterilization usually increases slightly the rapidity and the completeness of its bacteriolytic action (table I). It is possible that a more pronounced phenomenon of this nature is the phenomenon described by Kling and others as an activation of the extract.

The addition of larger amounts of inactive serum invariably leads to a more or less complete inhibition of the bactericidal properties (table II). Sera differ considerably in the amount of this antibactericidal action, rabbit serum, for example, having approximately four times the antibactericidal power of horse serum.

TABLE II.

*Effect of Inactive Serum on the Bacteriolysin.*

The tests were made as in table I, but with larger amounts of the inactive serum. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.				
	1 min.	1 hr.	2 hrs.	5 hrs.	24 hrs.
Bacteriolysin alone.....	600	6	0	0	0
Bacteriolysin + 1.5% inactive serum.....	600	5	0	0	0
Bacteriolysin + 3% inactive serum.....	800	20	2	0	30
Bacteriolysin + 6% inactive serum.....	850	50	10	0	2,000
Bacteriolysin + 12% inactive serum.....	850	400	250	200	3,000
Bacteriolysin + 25% inactive serum.....	950	550	500	400	4,000
Bacteriolysin + 50% inactive serum.....	1,020	850	820	800	16,000
100% inactive serum alone.....	1,050	1,000	950	1,020	20,000

*Active Serum.*—The effects of the addition of active serum are less easily determined, due to the bactericidal properties of the serum itself. By carefully adjusting the relative amounts of serum and extract, however, it is possible to show (table III) that active serum also is capable of inhibiting or destroying the bacteriolytic action. But what from the practical point of view is possibly of equal importance is the fact that the active serum as well as the extract loses its bactericidal power, as a result of such admixture. This gives the phenomenon of two bactericidal substances, active serum and active leucocytic extract, added to each other to produce a fairly good culture medium for bacteria.

TABLE III.

*Effect of Active Serum on the Bacteriolysin.*

Guinea pig serum was selected on account of its comparatively weak bactericidal action on *B. typhosus*. Tests as in table I. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	1 hr.	3½ hrs.	24 hrs.
10% guinea pig serum alone.....	1,300	450	0	0
Bacteriolysin alone.....	450	5	0	0
Bacteriolysin + 10 % guinea pig serum .....	1,200	1,000	750	15,000

EFFECT OF NORMAL TISSUE FLUIDS ON THE BACTERIOLYSIN.

Cerebrospinal fluid was selected as the easiest obtainable normal tissue fluid. The addition of cerebrospinal fluid to leucocytic extract produces nearly as great an inhibition or destruction of its bacteriolytic properties as the addition of serum itself (table IV).

TABLE IV.

*Effect of Cerebrospinal Fluid on the Bacteriolysin.*

Human cerebrospinal fluid (apparently normal) obtained by lumbar puncture for diagnostic purposes was tested as in table II. Test organism, *B. typhosus*. Dilutions made with 0.22 per cent. sodium chloride. A similar action was obtained with the cerebrospinal fluid of the monkey and the dog.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin alone.....	300	1	0	0
Bacteriolysin + 2% cerebrospinal fluid.....	350	0	0	0
Bacteriolysin + 4% cerebrospinal fluid.....	700	20	1	0
Bacteriolysin + 8% cerebrospinal fluid.....	750	250	4	3,000
Bacteriolysin + 16% cerebrospinal fluid.....	800	300	250	10,000
Bacteriolysin + 33% cerebrospinal fluid.....	1,000	600	700	15,000
Bacteriolysin + 66% cerebrospinal fluid.....	1,000	750	800	25,000
100 % cerebrospinal fluid alone.....	1,000	1,000	1,200	20,000

EFFECTS OF PATHOLOGICAL FLUIDS ON THE BACTERIOLYSIN.

*Pathological Effusion.*—The action of a pathological effusion is also practically the same as that of serum (table V).

TABLE V.

*Effect of Pathological Effusion on the Bacteriolysin.*

The effusion here tested was obtained by injecting aleuronat into the pleural cavity of a dog. The resulting pleural exudate was aspirated, twenty-four hours later, was centrifuged free from cellular elements, and then inactivated by heating it to 57° C., for twenty minutes. Tests of its antibactericidal action were made as in table II. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating			
	1 min.	45 min.	3½ hrs.	24 hrs.
Bacteriolysin alone.....	300	0	0	0
Bacteriolysin + 1.5% pleural effusion.....	450	3	0	0
Bacteriolysin + 3% pleural effusion.....	500	20	0	0
Bacteriolysin + 6% pleural effusion.....	650	50	1	10
Bacteriolysin + 12% pleural effusion.....	800	60	2	40
Bacteriolysin + 25% pleural effusion.....	1,000	500	250	6,000
Bacteriolysin + 50% pleural effusion.....	1,150	1,000	650	20,000
50% pleural effusion alone.....	1,100	1,100	900	2,000

*Autolytic Products.*—Substances given off during the aseptic autolysis of tissues are also strongly antibactericidal (table VI), as are also most of the products obtained by the bacterial decomposi-

TABLE VI.

*Effect of Autolytic Products on the Bacteriolysin.*

The kidney of a dog was removed with aseptic precautions, ground up in sterile sand, and the resulting finely divided tissue was washed three times by centrifugation with physiological saline solution. The serum-free tissue fragments were now suspended in three volumes of  $\frac{3}{4}$  physiological saline solution, and the resulting suspension was incubated over night. The suspension was then freed from tissue fragments by centrifugation and its antibactericidal powers were tested. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride. A similar antibactericidal action was obtained with the autolytic products of liver, spleen, and heart muscle.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin alone.....	600	20	0	0
Bacteriolysin + 0.75% autolytic products.....	850	100	0	0
Bacteriolysin + 1.5% autolytic products.....	850	150	6	0
Bacteriolysin + 3% autolytic products.....	850	350	150	25
Bacteriolysin + 6% autolytic products.....	900	500	350	600
Bacteriolysin + 12% autolytic products.....	950	600	400	2,000
Bacteriolysin + 25% autolytic products.....	950	700	600	15,000
Bacteriolysin + 50% autolytic products.....	980	850	850	30,000
50% autolytic products, alone.....	1,100	1,050	950	40,000

tion of tissues. None of the autolytic products thus far obtained have been in themselves bactericidal. A number of them have had the power of inhibiting bacterial multiplication, the property studied by Conradi.<sup>10</sup>

Of particular interest are the effects of products obtained by the prolonged autolysis of leucocytes themselves. On prolonged autolysis, both homologous leucocytes and foreign leucocytes give off strongly antibactericidal substances (table VII). The formation or liberation of these antibactericidal leucocytic products undoubtedly explains the fact, noted in the previous paper, that, in order to obtain an active leucocytic extract, the extraction process must be interrupted at a certain stage. Too prolonged an extraction usually gives an inactive extract.

TABLE VII.

*Effect of the Products of the Prolonged Autolysis of Leucocytes on the Bacteriolysin.*

Horse leucocytes were extracted at 37° C. for two hours in  $\frac{1}{4}$  physiological saline solution, and were then freed from the supernatant fluid by centrifugation. The partially extracted leucocytes were now suspended in three volumes of  $\frac{1}{4}$  physiological saline solution and the autolysis was allowed to continue at 37° C. over night. The resulting autolytic products were then freed from cellular elements and tested for their antibactericidal action. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	45 min.	3½ hrs.	24 hrs.
Bacteriolysin alone .....	600	50	0	0
Bacteriolysin + 0.5% leucocytic products .....	750	60	0	0
Bacteriolysin + 1% leucocytic products .....	800	50	0	0
Bacteriolysin + 2% leucocytic products .....	800	150	0	5
Bacteriolysin + 4% leucocytic products .....	800	600	200	15
Bacteriolysin + 8% leucocytic products .....	750	700	350	300
Bacteriolysin + 16% leucocytic products .....	800	600	400	500
Bacteriolysin + 33% leucocytic products .....	950	850	500	1,000
Bacteriolysin + 66% leucocytic products .....	950	850	750	2,000
66 % leucocytic products alone. ....	850	750	800	2,000

#### QUANTITATIVE RELATIONS.

*Amount.*—The relation between the amount of bacteriolysin tested and the amount of serum or other body fluids necessary to

<sup>10</sup> Conradi, H., *loc. cit.*, p. 222.

produce a given decrease in its bactericidal power is indicated in table VIII.

From this table it is seen that the amount of serum necessary to produce a given change in the bactericidal power increases with the amount of the bacteriolysin tested.

TABLE VIII.  
*Quantitative Relations.*

Parallel tests of the antibactericidal power of horse serum, with three different amounts of the bacteriolysin. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Serum necessary to reduce bacteriolysis.				Average.
	Slightly.	Markedly.	Nearly completely.	Completely.	
0.25 c.c. bacteriolysin.....	3%	5%	7%	11%	6.5%
0.35 c.c. bacteriolysin.....	7%	11%	16%	22%	14.0%
0.50 c.c. bacteriolysin.....	7%	16%	22%	33%	19.5%

*Rate.*—The neutralization, destruction, or binding of the bactericidal substance takes place instantaneously on the addition of the serum or body fluid. The reaction differs in this particular from a number of serum reactions, which require a certain length of time before becoming complete. Duplicate mixtures of serum and bacteriolysin tested immediately and after standing for various lengths of time show, within the limits of the experimental error, the same bactericidal powers.

#### ANTIBACTERICIDAL ACTION ANALYZED.

An effort was made to determine which of the components of serum and body fluids are responsible for the antibactericidal action.

*Serum Colloids.*—Serum colloids were obtained by dialyzing serum free from diffusible products. The precipitated globulins were put in solution by the addition of a minimum known amount of sodium chloride, and in testing the antibactericidal action the same amount of sodium chloride was used in all parallel and control tubes. The results of the analysis are shown in table IX. From this table it appears that about half the antibactericidal action of serum is due to the serum colloids.

TABLE IX.

*Effect of the Serum Colloids on the Bacteriolysin.*

The antibactericidal action of inactive horse serum was compared with that of the colloids obtained from the same serum. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin alone.....	950	60	0	0
Bacteriolysin + 2% whole serum.....	950	50	0	0
Bacteriolysin + 4% whole serum.....	700	0	0	0
Bacteriolysin + 8.5% whole serum.....	600	6	0	50
Bacteriolysin + 17% whole serum.....	800	350	70	8,000
Bacteriolysin + 33% whole serum.....	1,100	1,000	800	10,000
Bacteriolysin + 66% whole serum.....	1,150	1,000	1,000	30,000
Bacteriolysin + 2% serum colloids.....	900	2	0	0
Bacteriolysin + 4% serum colloids.....	700	15	0	0
Bacteriolysin + 8.5% serum colloids.....	600	1	0	0
Bacteriolysin + 17% serum colloids.....	600	30	0	60
Bacteriolysin + 33% serum colloids.....	700	400	100	1,200
Bacteriolysin + 66% serum colloids.....	1,050	650	750	2,000
66% whole serum alone.....	1,100	1,000	1,050	25,000
66% serum colloids alone.....	1,200	1,100	1,100	20,000

*Serum Crystalloids.*—The diffusible serum components were obtained by collecting the water from the dialysis above, and evaporating it to a small volume. The action of these diffusible products is shown in table X. This table confirms the previous finding, since

TABLE X.

*Effect of the Serum Crystalloids on the Bacteriolysin.*

The serum crystalloids, separated by dialysis from the serum colloids tested in table IX, were tested for their antibactericidal action. Test organism, *B. typhosus*. Dilutions made with 0.2 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin alone.....	950	60	0	0
Bacteriolysin + 2% serum crystalloids.....	600	4	0	0
Bacteriolysin + 4% serum crystalloids.....	1,000	150	10	0
Bacteriolysin + 7.5% serum crystalloids.....	1,000	100	2	0
Bacteriolysin + 15% serum crystalloids.....	600	50	0	2
Bacteriolysin + 30% serum crystalloids.....	800	120	30	3,000
Bacteriolysin + 60% serum crystalloids.....	950	700	700	30,000
60% serum crystalloids alone.....	1,100	1,050	1,100	20,000

it shows that approximately half the antibactericidal action of serum is due to its non-colloidal or diffusible components. The fact that the diffusible serum components possess a considerable antibactericidal action agrees with the observation that cerebrospinal fluid is strongly antibactericidal. Normal cerebrospinal fluid contains only a trace of colloidal (proteid) material, but is rich in diffusible serum components.<sup>11</sup>

**Ringer Solution.**—The action of Ringer solution is similar to the action of the diffusible serum products (table XI).

TABLE XI.

*Influence of Ringer Solution on the Bacteriolysin.*

Equal amounts of the bacteriolysin were dissolved in distilled water, half strength Ringer solution, and full strength Ringer solution, and then tested for their bactericidal power. The Ringer solution used in these tests had the following composition: 2.5 c.c. *m/l*  $\text{NaHCO}_3$  + 2 c.c. *m/l*  $\text{CaCl}_2$  + 4 c.c. *m/l*  $\text{KCl}$  + 9 gm.  $\text{NaCl}$  + 990 c.c.  $\text{H}_2\text{O}$ . Test organism, *B. typhosus*.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin in distilled water.....	500	30	0	0
Bacteriolysin in 50% Ringer solution.....	800	150	20	15,000
Bacteriolysin in 100% Ringer solution.....	950	600	500	40,000

**Sodium Chloride.**—It was pointed out in the previous paper that sodium chloride is antibactericidal for leucocytic extract.<sup>12</sup> Its antibactericidal action, however, is distinctly less than that of

<sup>11</sup> For analyses of normal and pathological cerebrospinal fluids, see Mott, F. W., *Lancet*, 1910, ii, 1, 79.

<sup>12</sup> The fact that an extract of horse leucocytes is decreased in its bactericidal power by the addition of sodium chloride does not agree with Schattenfroh's original observation (*loc. cit.*) that the action of the leucocytic bacteriolysin is independent of the salt content of the fluid tested. Schattenfroh's observation is of considerable importance, because it was cited as evidence that the leucocytic bacteriolysin and the serum bacteriolysin are two distinct substances, the bactericidal properties of serum being influenced by variations in its salt content.

The fact that an extract from horse leucocytes changes its bactericidal power on altering the amount of sodium chloride it contains does not, however, furnish evidence of the identity of the serum and leucocytic bacteriolysins. The serum bacteriolysin is active in the presence of sodium chloride, but inactive in distilled water. The leucocytic bacteriolysin is active in distilled water, but almost

Ringer solution (table XII). The antibactericidal action of sodium chloride is not due to a destruction or permanent injury of the bacteriolysin, since on removing the sodium chloride by dialysis the bactericidal power is restored quantitatively.

TABLE XII.

*Comparative Antibactericidal Action of Physiological Saline Solution and Ringer Solution.*

Equal amounts of the bacteriolysin were dissolved in distilled water, physiological saline solution (0.9 per cent. sodium chloride), and Ringer solution, and then tested for their bactericidal power. Test organism, *B. typhosus*.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin in distilled water .....	1,000	350	0	0
Bacteriolysin in physiological saline solution.....	1,000	600	400	1,500
Bacteriolysin in Ringer solution.....	1,000	900	900	5,000

*Alkalies.*—The addition of alkali to the bacteriolysin causes a rapid decrease in its bactericidal power (table XIII). The bacteriolytic action, for example, is nearly completely abolished in the

TABLE XIII.

*Influence of Alkali on the Bacteriolysin.*

Equal amounts of the bacteriolysin were tested alone and in the presence of increasing amounts of sodium hydroxide. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4 hrs.	24 hrs.
Bacteriolysin alone .....	650	100	2	10
Bacteriolysin + 0.0004% sodium hydroxide.....	800	200	20	50
Bacteriolysin + 0.0008% sodium hydroxide.....	760	250	30	70
Bacteriolysin + 0.0016% sodium hydroxide.....	860	550	300	350
Bacteriolysin + 0.0033% sodium hydroxide.....	780	500	600	550
Bacteriolysin + 0.0067% sodium hydroxide.....	750	520	500	400
0.0067% sodium hydroxide alone.....	770	600	600	600

inactive in the presence of sodium chloride. The observation therefore tends to strengthen Schattenfroh's conclusion that the two lysins are distinct. It is probable that the differences in the results are due to differences in the methods of preparing the leucocytic extracts.



presence of 0.003 per cent. sodium hydrate. A similar, though possibly slightly less pronounced inhibition is produced by sodium carbonate. The antibactericidal action of alkalis is not due to a destruction or permanent injury of the bactericidal agent, since on neutralizing an inactive mixture of bacteriolysin and alkali the bactericidal power is restored quantitatively.

*Acids.*—The determination of the effects of acids on the bacteriolysin is limited by the toxicity of most acids. Hydrochloric acid, within the limits of its toxicity, apparently has no effect. The range of the test is somewhat greater with acetic acid (table XIV), but within the limits of the experimental error, acetic acid also is without a distinct action on the leucocytic bacteriolysin.

TABLE XIV.

*Influence of Acetic Acid on the Bacteriolysin.*

Equal amounts of the bacteriolysin were tested alone and in the presence of increasing amounts of acetic acid. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4 hrs.	24 hrs.
Bacteriolysin alone.....	650	100	2	7
Bacteriolysin + 0.0003% acetic acid.....	750	90	2	8
Bacteriolysin + 0.0006% acetic acid.....	800	100	4	50
Bacteriolysin + 0.0012% acetic acid.....	900	90	2	30
Bacteriolysin + 0.0025% acetic acid.....	700	60	6	0
Bacteriolysin + 0.0050% acetic acid.....	650	60	3	0
0.0050% acetic acid alone.....	1,000	850	750	700

Boric acid is less toxic for bacteria than acetic acid, and can be tested in much higher concentrations (table XV). When added to leucocytic extract in small amounts, boric acid also is without any distinct action on bacteriolysis. It is only when the amount reaches 0.2 per cent. that a slight slowing in the rate of the bacteriolysis is observed. It is questionable, however, whether this is really due to acid action, or may not result from the necessary changes in osmotic condition. With 1.25 per cent. boric acid, bacteriolysis is distinctly reduced.

TABLE XV.

*Influence of Boric Acid on the Bacteriolysin.*

Equal amounts of the bacteriolysin were tested alone and in the presence of increasing amounts of boric acid. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride.

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4 hrs.	24 hrs.
Bacteriolysin alone.....	550	30	0	0
Bacteriolysin + 0.025% boric acid.....	450	1	0	0
Bacteriolysin + 0.05% boric acid.....	700	5	0	0
Bacteriolysin + 0.1% boric acid.....	800	40	0	0
Bacteriolysin + 0.2% boric acid.....	800	300	6	0
Bacteriolysin + 0.4% boric acid.....	750	300	3	0
Bacteriolysin + 0.8% boric acid.....	750	300	0	0
Bacteriolysin + 1.25% boric acid.....	750	400	100	20
Bacteriolysin + 2.5% boric acid.....	800	300	200	50
2.5% boric acid alone.....	750	750	700	650

*Quantitative Relations.*—The relative amount of the destruction or inhibition of bacteriolysis that is due to each of the above components has not been determined. With serum, however, it is probable that about half the antibactericidal action is due to the serum colloids, about a quarter to the neutral diffusible products, and a quarter to the diffusible alkalies.

## MECHANISM OF THE ANTIBACTERICIDAL ACTION.

The manner in which the various substances above enumerated antagonize or overcome the bactericidal action has not been determined. Two methods are theoretically possible. First, the antibactericidal substances may enter into a direct chemical relation with the bacteriolysin, with the formation of non-bactericidal combination products or split products. Or second, the antibactericidal substance may enter into direct chemical relation with the bacteria, making them in some way insusceptible to the action of the bacteriolysin. This second method might conceivably be somewhat similar to the antagonistic salt actions studied by Loeb<sup>18</sup> and his co-workers, in which one chemical substance so alters the permeability of the limiting cell membrane as to prevent the entrance into the cell of the toxic agent. It is quite possible, and even probable,

<sup>18</sup> Loeb, J., *The Dynamics of Living Matter*, New York, 1906, 70.

that the antagonistic action of any one of the antibactericidal body fluids is a combination of a direct action of certain components on the bacteriolysin and of other components on the bacteria.

#### METHODS TO PREVENT THE ANTIBACTERICIDAL ACTION.

*Quantity.*—An attempt was made to devise a method to overcome or prevent the antibactericidal action of serum and body fluids. The first method that suggested itself was to exhaust the antibactericidal power of the inhibiting fluid by increasing the amount of bacteriolysin added to it. It is possible, under certain conditions, to overcome the antibactericidal action of sodium chloride by this means (table XVI).

TABLE XVI.

##### *Attempt to Exhaust the Antibactericidal Action of a Neutral Salt.*

Parallel tests with multiple doses of the leucocytic bacteriolysin dissolved in physiological saline (0.9 per cent. sodium chloride). Test organism, *B. typhosus*.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin in distilled water .....	650	40	5	0
Bacteriolysin in physiological saline.....	600	550	600	25,000
2 X bacteriolysin in physiological saline.....	600	350	300	20,000
4 X bacteriolysin in physiological saline.....	400	10	5	2,000
8 X bacteriolysin in physiological saline.....	400	5	0	0

It is also possible to overcome part of the antibactericidal action of Ringer solution by the same means (table XVII).

TABLE XVII.

##### *Attempt to Exhaust the Antibactericidal Action of Ringer Solution.*

Parallel tests with multiple doses of the leucocytic bacteriolysin dissolved in Ringer solution. Test organism, *B. typhosus*.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin in distilled water .....	1,000	70	1	0
Bacteriolysin in Ringer solution.....	1,000	950	1,000	50,000
2 X bacteriolysin in Ringer solution.....	1,000	700	600	50,000
4 X bacteriolysin in Ringer solution.....	1,000	300	70	20,000
8 X bacteriolysin in Ringer solution .....	950	150	20	2,500

TABLE XVIII.

*Attempt to Exhaust the Antibactericidal Action of Body Fluids.*

Parallel tests with multiple doses of the leucocytic bacteriolysin dissolved in human cerebrospinal fluid. Test organism, *B. typhosus*. Dilutions made with 0.22 per cent. sodium chloride. A practically identical result was obtained with inactivated pathological transudate and with inactive serum.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin in distilled water . . . . .	1,100	350	0	0
Bacteriolysin in cerebrospinal fluid . . . . .	1,100	1,000	1,000	6,000
2 X bacteriolysin in cerebrospinal fluid . . . . .	1,050	1,000	1,000	8,000
4 X bacteriolysin in cerebrospinal fluid . . . . .	1,050	950	1,000	8,000
8 X bacteriolysin in cerebrospinal fluid . . . . .	1,100	1,000	1,000	6,000

Attempts to overcome the antibactericidal action of cerebrospinal fluid (table XVIII), of pathological transudates, and of serum by this means, however, have thus far been unsuccessful. Apparently no amount of leucocytic bacteriolysin added to these fluids, when tested in their full concentration, is able to exhaust their antibactericidal capacity. If the fluids, however, are diluted, their action is more nearly that of Ringer solution.

TABLE XIX.

*Attempt to Diminish the Antibactericidal Action of Body Fluids.*

Parallel samples of a non-bactericidal mixture of the leucocytic bacteriolysin and serum were tested alone and in the presence of increasing amounts of boric acid. Test organism, *B. typhosus*. Test fluids contained 0.22 per cent. sodium chloride. A practically identical result was obtained with a non-bactericidal mixture of the bacteriolysin and cerebrospinal fluid. A similar restoration of part of the original bactericidal power can be obtained with acetic acid.

Material tested.	Time of plating.			
	1 min.	1 hr.	4 hrs.	24 hrs.
Bacteriolysin alone . . . . .	1,200	60	0	0
Bacteriolysin + serum . . . . .	1,100	1,050	1,100	50,000
Bacteriolysin + serum + 0.017% boric acid . . . . .	1,050	1,000	1,000	8,000
Bacteriolysin + serum + 0.035% boric acid . . . . .	1,100	900	850	6,000
Bacteriolysin + serum + 0.07% boric acid . . . . .	1,050	850	800	1,200
Bacteriolysin + serum + 0.15% boric acid . . . . .	1,050	750	800	850
Bacteriolysin + serum + 0.3% boric acid . . . . .	1,000	600	600	350
Bacteriolysin + serum + 0.6% boric acid . . . . .	1,000	600	600	400
Bacteriolysin + serum + 1.25% boric acid . . . . .	1,100	550	500	350
Bacteriolysin + serum + 2.5% boric acid . . . . .	1,050	600	400	300

**Acidulation.**—A second method that suggested itself was to neutralize the antibactericidal alkalies. This method is partially successful. The addition, for example, of boric acids to a non-bactericidal mixture of the bacteriolysin and body fluids occasionally restores part of the original bactericidal power (table XIX). In no case thus far tested, however, has more than a small fraction of the original power been restored by this means. The result differs in this respect from the result reported by Lamar,<sup>14</sup> who was able to restore completely the inhibited bactericidal power of certain soaps by adding boric acid to an inactive mixture of the soap and serum.

#### SUMMARY.

1. An extract of horse leucocytes is strongly bactericidal when dissolved in distilled water; it has considerable bactericidal power when dissolved in physiological saline; but it loses its bactericidal properties when mixed with blood serum or with normal or pathological tissue fluids.

2. About half the antibactericidal action of blood serum is due to the serum colloids, about a quarter to the neutral serum crystalloids, and a quarter to the diffusible alkalies. Diffusible acids have no antibactericidal action.

3. The addition of boric acid to an inactive mixture of leucocytic extract and serum or other body fluid occasionally restores part of the original bactericidal power, but never more than a small fraction of that power.

<sup>14</sup> Lamar, R. V., *Jour. Exper. Med.*, 1911, xiii, 1, 380; xiv, 256.

## A TRANSPLANTABLE NEW GROWTH OF THE FOWL, PRODUCING CARTILAGE AND BONE.\*

By W. H. TYTLER, M.B.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

PLATES 79 TO 83.

Neoplasms of the common fowl have been described by numerous authors, and include tumors of various classes. Up to the present time four transplantable tumors of the fowl have been reported, one of which is the growth here dealt with. Fujinimi and Inamoto in 1910 reported at a meeting in Japan a myxosarcoma which they had succeeded in transferring to other fowls.<sup>1</sup> In the same year Rous<sup>2</sup> described the transplantation of a spindle-celled sarcoma which at times showed myxomatous tendencies. This tumor was highly malignant, usually leading to the death of the animal, and often producing widespread metastases. The tumor reported by Fujinimi and Inamoto appears to have been not so malignant, though sometimes metastasizing and causing death. The tumor which forms the subject of the present paper is an osteochondrosarcoma and has been mentioned in an earlier note<sup>3</sup> from this laboratory, in which was also reported the fourth transplantable chicken tumor, a spindle-celled sarcoma. For purposes of reference the osteochondrosarcoma is known in this laboratory as Chicken Tumor VII. It has great interest from at least two points of view. Its cells regularly undergo a process of metaplasia and differentiation to form cartilage and bone; and the growth is transmissible, like the spindle-celled sarcoma of Rous, by an agent separable from the tumor tissue and filterable through Berkefeld filters.<sup>4</sup> The filtrate,

\* Received for publication, October 26, 1912.

<sup>1</sup> Fujinimi, A., and Inamoto, K., *Verhandl. d. Jap. path. Gesellsch.*, 1911, 114.

<sup>2</sup> Rous, P., *Jour. Exper. Med.*, 1910, xii, 696; 1911, xiii, 397.

<sup>3</sup> Rous, P., Murphy, J. B., and Tytler, W. H., *Jour. Am. Med. Assn.*, 1912, lviii, 1682.

<sup>4</sup> Rous, P., Murphy, J. B., and Tytler, W. H., *Jour. Am. Med. Assn.*, 1912, lix, 1793.

when brought into contact with connective tissue under suitable circumstances, gives rise to the characteristic growth in which is laid down cartilage, followed often by bone.

#### DESCRIPTION OF THE ORIGINAL GROWTH.

The fowl bearing the original growth was obtained while yet alive from a dealer in poultry. It was a Plymouth Rock hen, in good condition, of approximately pure breed, and apparently about a year old. On the lower portion of the keel of the sternum was an irregularly spherical mass, so symmetrically disposed that the keel passed almost through its center. It measured 6.6 by 5.7 by 4 centimeters. The growth was smooth, nearly as hard as bone, and the skin over it was slightly stretched but not firmly attached (figure 1). A wedge-shaped piece of it was taken out under ether anesthesia. The tissue thus obtained was found to be firm, white, and fibrous, with some translucent areas suggesting cartilage. Small bits were at once implanted in the original fowl, by means of a trocar (Bashford needle). Two such grafts were placed in the pectoral muscles, and four subcutaneously, on each side.

An examination of the fowl thirty-six days later showed that the original mass had not grown, except perhaps at the edges of the recent incision. The gap left by the removal of tissue had persisted almost unchanged, and was filled with purulent material. At six of the ten inoculation sites small nodules were felt. The fowl was in poor condition, so it was killed, and bits of the tumor tissue were inoculated into twenty young, pure-bred Plymouth Rock hens, the trocar again being used. Two of these died of intercurrent disease, but in twelve of the eighteen that survived growths developed, appearing in general within thirty days. The propagation of the tumor has since been easy, and it is now in its seventh transplantation generation.

#### GENERAL MORPHOLOGY OF THE GROWTH.

The general morphology of the transplantation tumors does not differ from that of the original or spontaneous growth. The latter was removed at autopsy together with the lower half of the

sternum, and was cut transversely. It was well encapsulated. In the gross specimen the sternal keel could be traced to the growth's center, but here it was lost in a mass of red, spongy, bony tissue which radiated from it a distance of one to one and one half centimeters (figure 2). Peripheral to this the tumor was hard and white, with fine strands of opaque, fibrous tissue separating more translucent, homogeneous areas. The secondary nodules, globular and well defined, were composed of the opaque, fibrous tissue. The fowl was emaciated. No metastases were found.

Microscopically the capsule of the original growth was found to consist of fibrous connective tissue containing isolated muscle bundles. The growth itself was made up of a zone of what may be termed prechondral tissue, enclosing and grading into a mass of hyaline cartilage through which ran the sternal keel. From this latter numerous irregular, bony trabeculae radiated into the cartilage.

#### HISTOLOGICAL FINDINGS.

The outer, or prechondral, tumor tissue is an irregular connective tissue composed of sparsely scattered cells in a relatively large amount of fibrillar intercellular substance. The cells closely resemble normal connective tissue cells of the young fibroblast type, having large vesicular nuclei, some with two nucleolar masses and some showing amitotic division or nuclear budding. The cells lie, singly or in groups, in slit-like spaces, and in the groups are larger and more globular. Blood vessels are numerous, and their walls consist usually of endothelium only. The intercellular substance gives the staining reaction for collagen.

Deeper in are occasional islands in which the cells are polyhedral or globular, and lie in irregular spaces suggesting capsules, while the intercellular substance is here relatively homogeneous, though non-staining. These islands strongly suggest cartilage, and in fact all transitions are found between them and actual cartilaginous areas lying further toward the center of the tumor. The cartilage cells are globular, in well formed capsules, and the ground substance stains deep blue with hematoxylin, taking an intense blue at the capsule rims. With aniline blue the ground substance takes a light, diffuse stain and also shows numerous fine, deeply staining, collagen fibrils.

Near the center of the growth the cartilage undergoes a transition to a non-calcified, osteoid tissue, which itself undergoes calcification and partial resorption. It is in this way that the radiating bony trabeculae already mentioned appear to have been formed. The deeply staining cartilage masses, at their inner border, shade into a zone with a non-staining, homogeneous ground substance, the cartilage cells at the same time showing various stages of transition to cells with the general morphology of bone corpuscles (figure 7). Still further



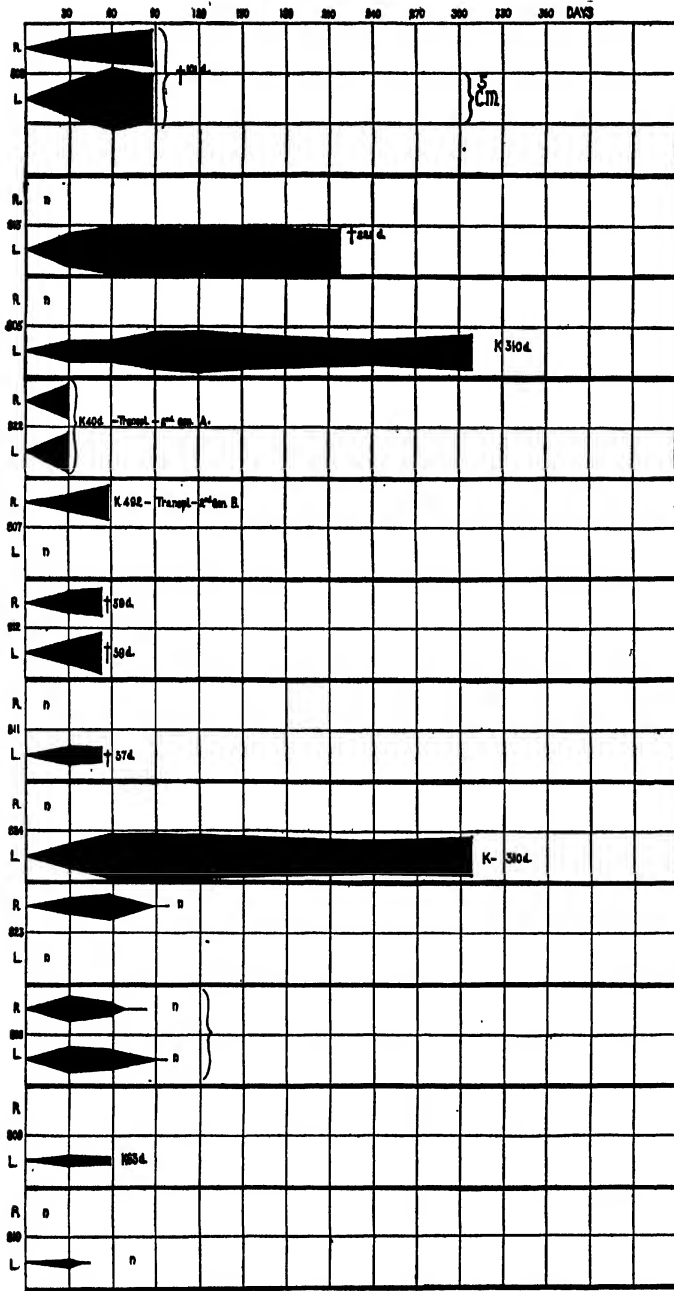
in, calcification is taking place with the formation of trellis-like figures, staining deep blue with hematoxylin, between the cells of the osteoid tissue. Into this calcified zone, which in most places is quite narrow, marrow spaces, with their blood vessels and osteoclasts, are advancing, and resorption is taking place. Thus are formed irregular bony trabeculae. The intertrabecular marrow spaces contain a loose connective tissue, for the most part only sparsely cellular, but in places packed with cells that resemble fibroblasts. Occasionally small collections of granular leucocytes, mostly of the myelocyte type, are seen. Blood vessels are small and not numerous. Deep in the mass the keel of the sternum is recognizable as two roughly parallel bony plates (figure 4). The bony trabeculae which radiate from the outer surface of these into the tumor tissue closely resemble the trabeculae of the medullary cavity. The periosteum can be traced from the base of the sternum up to the lowest of these bony processes on each side, but here it is lost in tumor tissue. Toward its free edge the sternal keel is broken through in many places and here the newly formed bone is continuous with the medullary trabeculae. The edges of the gaps show active erosion by osteoclasts.

The secondary subcutaneous and intramuscular nodules consist practically entirely of prechondral tissue of the sort described. This tissue is much more cellular than in the original tumor, the cells lying close together with but a small amount of intercellular substance. Many of them have two nucleoli, and some show amitotic division in progress, or, more rarely, a mitotic figure may be seen. At its margin the prechondral tissue extends out between the muscle bundles, while external to this there is, amid the muscle, a diffuse increase of connective tissue.

To recapitulate briefly: The outermost, youngest zone of the tumor is made up of cells of fibroblastic type scattered sparsely in a collagenous intercellular substance. In its deeper portions this tissue is undergoing a transformation to cartilage, its cells taking the character of cartilage cells, while the ground substance becomes homogeneous and basic staining. The original growth is well encapsulated, and seems nearly, if not quite, stationary. The secondary tumors have grown rapidly, are more cellular than the primary growth, and are invasive in tendency.

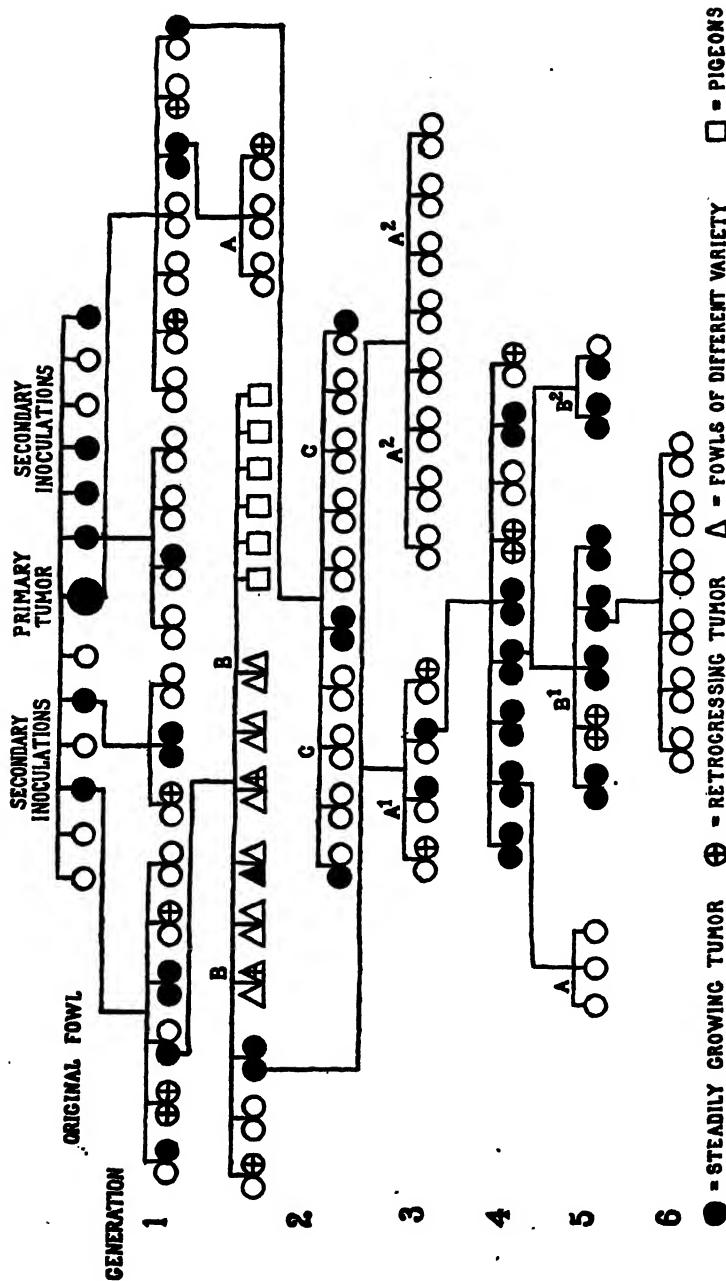
#### GENERAL RESULTS OF TRANSPLANTATION.

The growth has been propagated in series of chickens by transplanting small bits of the fresh, peripheral, neoplastic cartilage. In general, Plymouth Rock chickens have been used, and by means of a trocar the grafts have been placed deep in the pectoral muscles. In one series of four fowls prechondral tumor tissue was placed in one breast, in the other cartilage from the same tumor. Growths



TEXT-FIG. 1. Chart showing the results of implantations in the right and left pectoral regions of the twelve susceptible fowls of the first transplantation generation. The tumor growth is represented as a black band, the thickness of the band being that of the average diameter of the growth at any one time, and its length that of the number of days that the growth endured. The angular outlines of the bands are referable to the fact that they were plotted from measurements taken at considerable intervals. Rapid growth followed by slowing or retrogression is characteristic of Chicken Tumor VII.

From the figure it would appear that the tumors began to grow immediately on the implantation of the grafts. This was probably not the case, but a more correct chart cannot be made since the first examination did not take place until thirty days after the implantation. 809, 810, etc. = index number of the individual fowl. R and L = right and left pectoral regions.



developed in all four hosts, but only from the cartilage. The results of the transplantation from the original fowl are shown in text-figure 1, which also illustrates well the general behavior of all the propagated growths. The varying success of the tumor in its first five transplantation generations is shown in text-figure 2.

The percentage of successful inoculations from the primary growth was very high,—60 per cent. of the fowls yielding tumors and growths developing at 40 per cent. of all the inoculation sites. The first transplantation of spontaneous mouse tumors does not ordinarily yield nearly so high a proportion of growths. It is interesting to note that in Haaland's<sup>5</sup> series of seventy-eight spontaneous mouse tumors that were transplanted, the greatest average success was obtained with growths that on transplantation showed a general tendency to retrogress. In the case of Chicken Tumor VII this tendency is very marked.

Passage from host to host has scarcely increased the transplantability of the chicken tumor (text-figure 2). Furthermore, at present (the seventh tumor generation) it grows little if any more rapidly than when first transferred. But the prechondral tissue frequently infiltrates the surrounding muscle; and in one recent case a metastasis was found. For these reasons the tumor is best considered as an osteochondrosarcoma.

The development of Chicken Tumor VII after the implantation of a graft follows certain regular lines. The immediate fate of the implanted tissue has not yet been traced, but where it is placed in susceptible fowls there appears after ten days or two weeks a discrete, smooth, firm nodule which usually enlarges rapidly. It is ellipsoidal with its long axis in the direction of the muscle fibers. In one instance a mass four centimeters in diameter developed within thirty days after the implantation of a tumor bit not larger than four by one millimeters. There is often some invasion of the tissue surrounding such a nodule, but its growth is predominantly expansive in character and often it can be shelled out of its capsule, which at this time is usually well developed. Microscopically the tumor is found to consist of the peculiar prechondral tissue that

<sup>5</sup> Haaland, M., *Fourth Scientific Report of the Imperial Cancer Research Fund*, 1911, 1.

has been described in connection with the original growth. Cartilage is already forming at its center, as islands which gradually enlarge and merge into a mass that seems homogeneous to the naked eye, and as the mass grows larger degenerative changes occur at its center. With the gradual transformation to cartilage there takes place a slowing of the tumor's growth rate (text-figure 1), and after about fifty days nearly all the tumors become stationary, and many begin to retrogress. In some of the growing tumors, the proliferation of the prechondral rim and its transformation to cartilage slowly continue. If the host lives long enough ossification occurs within the cartilage and nearly the whole tumor may be converted into bone. The bony change takes place quite independently of whether the tumor is growing, stationary, or retrogressing, but in any event bone does not appear in the growth until the third or fourth month.

Instances in which this tumor has led to the death of the host are as yet few. Even when quite large it does not appear to cause ill health. At present there are in the laboratory numerous fowls which for many months have carried the growth in the breast as a stationary, retrogressing, or slowly growing lump of bony hardness. In the instances in which the tumor proved fatal the host has gradually emaciated and at length become comatose for a day or so before death. At autopsy no changes were found in the viscera other than those attendant upon emaciation and secondary anemia.

#### SPECIMEN PROTOCOLS.

*A Progressively Growing Tumor.*—Fowl 504, 7th generation A. In each breast of this fowl there was implanted a graft from No. 321, 6th generation A. In the right breast there soon developed a growth like a slightly flattened sphere, lying within the muscle, which increased rapidly in size up to the time of the fowl's death, sixty-seven days after the implantation. At autopsy the mass measured 6.4 by 5.6 by 4.5 cm. It projected sharply from the breast of the emaciated host. It was not attached to the sternum, but the pressure of its enlargement had arched inward the membrane stretching between two sternal ribs lying directly behind it. The growth was very firm and on section discrete, grayish white, of smooth, close texture, and translucent. Its center was honey-combed with blood channels, and showed at one point the old clot of a hemorrhage, at another a few bony spicules. Microscopically the growth consisted of hyaline cartilage with a thin rim of prechondral tissue. The central blood channels were walled only by endothelium. No metastases were found anywhere. It is doubtful if death was due to the tumor.

*A Metastasizing Tumor.*—Fowl 46, 2d generation C. A bit of tumor from No. 824, 1st generation A, was implanted in the muscle of each pectoral region of this fowl. Three weeks later a nodule about 1 cm. in diameter had developed at one site and after two months a similar growth appeared at the other. The tumors grew slowly and at length became stationary, remaining so for many weeks before the fowl's death which occurred from intercurrent causes seven months after the inoculation. At autopsy the tumors were found to be shaped like slightly flattened spheres. They measured 5 and 1.7 cm. respectively, in their greatest diameter, were discrete and stony hard, and consisted of a narrow, gristly rim of cartilage about a mass of spongy bone and red bone marrow.

On cutting away the sternum a characteristic tumor mass, 0.9 cm. in diameter, was disclosed, projecting sharply into the thoracic cavity and attached by a narrow base to the inner surface of the lowest right rib at the junction of its sternal and vertebral portions. Its situation was such that it could not be attributed to the primary implantation. It contained cartilage.

*A Stationary, Bony Tumor.*—Fowl 823, 1st generation A, and 6th generation A. This fowl was inoculated with two grafts of the original osteochondrosarcoma, and developed as a result one small tumor which was largely removed at operation, the remainder of it retrogressing. One hundred and five days after its disappearance a reinoculation was done, this time with material from No. 353, 5th generation B. A tumor developed which within a month reached a diameter of 2.5 cm., but then ceased to grow. Four months later, when the fowl was killed, it measured only 2 cm. across. At autopsy it proved to be a roughly spherical, bony nodule, with a smooth surface and a thin connective tissue capsule. It was made up of an outer shell or lamella of bone enclosing spongy bone and red marrow and a few islands of cartilage. The general health of the host had been good throughout.

#### HISTOLOGY OF THE METAPLASIA.

The formation of cartilage which takes place regularly in the transplanted growths (figures 5, 9, and 10) comes about in the same manner as in the original tumor. The islands of cartilage, at first isolated in prechondral tissue, gradually enlarge and become more numerous until to the naked eye their fusion appears complete. As a matter of fact, the microscope shows that they are still separated by thin strands of the prechondral tissue. In its interior the cartilage sooner or later undergoes degeneration, with loss of staining power and gradual death of the cells.

The change to bone also occurs in the same way as in the primary tumor. The cartilage undergoes a transformation to osteoid tissue, becomes calcified, and is actively eroded by osteoclasts to form trabeculae between which red bone marrow appears (figure 6). There is thus formed an irregular mass of spongy bone. The

process, as in the primary tumor, seems to be one of progressive resorption, with no production of new bone except as is obtained by the continuous conversion of cartilage into a calcified, osteoid tissue. Osteoblasts appear to play no part in the bone formation.

The bone cells are somewhat irregular in morphology, but the majority closely resemble normal bone corpuscles, being small, flattened, sharp angled, dark staining cells, lying in clear cut spaces. The ground substance is dense and homogeneous. The marrow spaces show large blood spaces, walled with endothelium and distended with red blood cells. Most of the red cells are not peculiar, but many are more globular than the normal and have a bluish grey cytoplasm (after eosin and methylene-blue) and pale, rounded, vesicular nuclei. Occasionally a mitotic figure is seen in one of these cells.

In the intervascular tissue are seen numerous spindle cells of the type seen at the growing border of the tumor. These cells occur also in more globular form, many with few or no cytoplasmic processes, but with the same large vesicular nuclei and pale staining cytoplasm as the prechondral cells. Small numbers of cells occur, however, of the same size and shape as these globular cells, and with the same type of nucleus, but with a strongly basophilic cytoplasm which may contain occasional eosinophilic granules. The nuclei occasionally show mitosis. These cells closely resemble the strongly basophilic cells seen in the development of human blood, and designated as *Wanderzellen*, myeloblasts, etc., by various authors (Maximoff, Askanazy, Schridde, Naegeli, and others). With these cells occur mononuclear cells filled with eosinophile granules, and the ordinary polymorphonuclear eosinophile leucocytes. These four types of cells, the globular, pale staining connective tissue cell, the basophilic cell with few or no granules, the myelocyte, and the polymorphonuclear leucocyte, are often seen lying together in nest-like collections, the last two types always being in excess. The suggestion is very strong that the myelocyte type takes its origin from the basophilic cell, which in turn seems to be related to the globular connective tissue cell. Whether these latter take their origin from tumor cells, representing thus another metaplastic change, has yet to be determined.

The striking characteristic of the retrogressing neoplasms is the replacement of tumor by fibrous connective tissue which from its maturity and uniformity would appear to be derived from the host tissues. The tumor is separated by this into small, sharply defined nodules, which consist of cartilage, or, in the case of the more outlying ones, of dense collagenous tissue that is still living. At the margin of the mass are many good sized blood vessels, surrounded usually by numerous lymphocytes. In old, stationary growths cartilage and prechondral tissue may practically disappear leaving a rounded, bony nodule consisting of a smooth outer lamella, or bony shell, and a spongy interior of trabeculae and red marrow.

#### INTRAVENOUS INOCULATION.

Three fowls of the 5th generation A received at the time of their original intramuscular inoculation an intravenous injection of a suspension of the finely ground tumor in Ringer solution. All fowls developed tumors in the breast muscle as a result of the inoculation at that site. Two of them showed at autopsy no visceral lesions. In the third fowl, No. 352, dying 136 days after the injection, there were found, in addition to the large, bony, pectoral growths, two tumor masses attached to the ovary, and each the size of a hen's egg, in which coils of small bowel were involved. There were also irregular disseminations of tumor tissue, up to 0.5 cm. in thickness, on the parietal and visceral surfaces of the peritoneum. Microscopically both the ovarian and the peritoneal growths showed characteristic prechondral tissue and cartilage. The ovarian tumors also showed extensive hemorrhage and necrosis. Whether these visceral growths resulted from the material injected intravenously, or were secondary to the pectoral tumors, cannot be definitely said. The relatively slight metaplastic changes observed in them favors the latter conjecture since it points to a recent origin for them.

#### BACTERIOLOGICAL EXAMINATION.

Smears taken from the tumor tissue and stained by various methods have failed to reveal any structures suggesting living organisms, as has also examination with the dark-field microscope.



Cultures were made on all the usual laboratory media, both aerobically and anaerobically. No growth resulted, save on two tubes which showed evident contamination.

#### IMMUNITY.

Several experiments have been made in the attempt to demonstrate the occurrence of an immunity, natural or acquired, against the tumor. The results obtained up to the present hardly seem to justify definite conclusions, owing partly to the comparatively small number of animals tested, and partly to a lack of uniformity in the material used for reinoculation, as shown by the results in control fowls. The following is a summary of the findings.

In the 3d generation B there were inoculated three Plymouth Rock hens, three black Minorca hens, three brown Leghorn hens, and six mongrel pigeons. No tumor growth took place in any of the pigeons, suggesting, for that species at least, the occurrence of a natural specific immunity, as is the case with animal tumors in general. Tumors developed, however, in both of the alien breeds of the same species as the original host. A difference of breed does not, then, constitute a barrier to the transplantation of Chicken Tumor VII. In this respect the tumor contrasts sharply with Chicken Tumor I (Rous<sup>6</sup>), which in its earlier generations could be transplanted only to fowls of close blood relationship to the original host.

Reinoculations have been made in several series of animals, including fowls which remained negative from the first inoculation, fowls bearing growing or retrogressing tumors, and fowls in which the growth had retrogressed completely. Reinoculation of fowls negative from the original implantation gave tumors only in one series of three fowls. These had been inoculated originally with tumor emulsion, which gave completely negative results. The other fowls of this class, twenty-two in all, still showed no growth after the second inoculation. Although the value of the result was lessened, for a part of this series, by the unsuitable nature of the material used for reinoculation, the findings suggest the occurrence of a strong natural immunity in certain individuals of the species.

<sup>6</sup> Rous, P., *loc. cit.*

In the case of fowls bearing tumors, or in which tumors had retrogressed, reinoculation gave a very small percentage of tumors as compared with the general results in normal fowls. The interpretation of the findings is complicated by several factors, which are important, one being the general poor health of fowls which have been confined for some time in close quarters. The inference is apparently justified, however, that a certain degree of resistance develops in animals in which the tumor has grown for any considerable period. If this is so it offers one explanation for the general tendency to retrogression which Chicken Tumor VII exhibits.

#### DISCUSSION.

The osteochondrosarcoma described occurred as a unique instance among some thirty primary chicken tumors brought to this laboratory during the last twelve months. It appears to have arisen from the periosteum, all the layers participating in the growth. Injury must be thought of as a contributory cause, because of the tumor's position. Its symmetrical arrangement and histological structure at first suggested that it was congenital in origin, but no evidence confirmatory to this view has been obtained.

In its general characters Chicken Tumor VII is like certain mouse tumors, also osteochondrosarcomata, described by Haaland<sup>7</sup> and Murray.<sup>8</sup> Murray's tumor was successfully transplanted and retained its ability to produce cartilage and bone. From his description it appears that these tissues were laid down in a spindle-celled parenchyma, much as happens in the case of Chicken Tumor VII. It is interesting in this connection to remember that dogs are not infrequently the subject of mixed tumors of the mamma which contain an epithelial element, cartilage, and bone, and occasionally give metastases showing one or more of these tissues. The development and differentiation which takes place in Chicken Tumor VII is somewhat similar to that occurring in the normal growth of the chick embryo, but in other ways is notably different. The tumor cells in their early form are relatively simple looking con-

<sup>7</sup> Haaland, M., *Ztschr. f. Krebsforsch.*, 1907, v, 125.

<sup>8</sup> Murray, J. A., *Third Scientific Report of the Imperial Cancer Research Fund*, 1908, 69.

nective tissue cells, and they become changed into elements which, save for the fact that they are hypertrophic, are wanting in none of the morphological characters of normal cartilage cells. At the same time an intercellular substance is formed which shows the morphological features and the staining reactions of the ground substance of normal cartilage. But between the connective tissue stage and the cartilage stage is an intermediate one during which the cell produces a large amount of collagenous material. Such a stage is not prominent in the development of the normal cartilage of the bird.

In many instances of Chicken Tumor VII the cartilage at length undergoes degeneration and death, possibly from insufficient blood supply. In others, or in other parts of the mass the cartilage is changed to an osteoid tissue which later undergoes calcification and erosion by osteoclasts, with resulting formation of bony trabeculæ. The bone formation takes place, as a rule, only in connection with the marginal healthy cartilage, so that a peripheral bony zone may be found enclosing a soft degenerated center (figure 3).

The process of bone formation differs in its order from that of normal endochondral bone in the chick, as described by Lillie,<sup>9</sup> for in the production of normal bone the cartilage is first calcified and then eroded, while at the same time osteoid tissue is deposited on the trabeculæ by osteoblasts. Whether in Chicken Tumor VII the bone marrow develops from neoplastic tissue or by an ingrowth of tissue elements from the host is unsettled, but the former process is suggested by the findings as an origin for many of the marrow elements, as is also the possibility of even certain of the blood cells having arisen from tumor tissue. Both hypotheses, of course, require more extensive confirmation.

#### SUMMARY.

An osteochondrosarcoma of the common fowl, designated in this laboratory as Chicken Tumor VII, has been successfully transplanted to seven successive series of hosts. The original growth contained bone and cartilage, was attached to the sternal keel of an otherwise healthy chicken, and appeared to have arisen from this

<sup>9</sup> Lillie, F. R., *The Development of the Chick*, New York, 1908, 409.

structure. In the growths derived from its transplantation cartilage is regularly laid down, followed later by bone in case the host lives long enough. The prechondral tissue consists of spindle-shaped or multipolar cells of the fibroblast type. The histological character and the behavior of this prechondral tissue show that it is sarcomatous, a fact further proven by one recent case in which the tumor has metastasized. The secondary growth in this instance consisted of prechondral tissue in which a cartilaginous change was taking place.

The tumor could not be transferred to pigeons, the one foreign species tested, but grew readily in chickens of two alien breeds. Reinoculation experiments suggest the occurrence of a natural, individual immunity, and of a certain degree of acquired resistance. In one fowl visceral growths developed following an intravenous injection of tumor emulsion, although whether they were due to this cause or were secondary to the large implantation growths in the muscles is uncertain. Recently the tumor has been transmitted by means of the filtrate from a Berkefeld filter.

#### EXPLANATION OF PLATES.

##### PLATE 79.

FIG. 1. Original tumor with a part of the sternal keel, viewed from the right side. The skin has been stripped away except over the crest of the keel. About three fourths natural size.

FIG. 2. Section through the center of the tumor and across the keel of the sternum. The keel lies vertically in the midst of the growth but is lost in a mass of spongy bone. A wedge-shaped piece of tissue has been cut from one side of the tumor. The dark areas deep in the growth are areas of spongy bone. *n* = the notch left by excision of the tissue; *k-k* = the position of the keel; *st* = the sternum at the base of the keel. About natural size.

##### PLATE 80.

FIG. 3. Cross section of a bony tumor resulting from transplantation. The growth was eighty-five days old and had been stationary for some weeks before its removal. In this instance a broad zone of spongy bony tissue surrounds a degenerated center. The growth is well encapsulated. About natural size.

FIG. 4. Original tumor. Microscopic preparations showing the disappearance of the sternal keel amid the growth's bony trabeculæ. At the upper left hand corner of the photograph is a dark mass of cartilage, which is separated from the bone by a layer of osteoid tissue nearly free from stain. *k-k* = lamellæ of the keel. Stained with hematoxylin.

## PLATE 81.

FIG. 5. Transplantation tumor (fowl 822, 1st generation). Duration of growth, thirty-five days. Microscopic preparation showing prechondral tissue and its transformation into cartilage. Stained with eosin and methylene-blue.

FIG. 6. Transplantation tumor (fowl 808, 1st generation), showing bony trabeculae, osteoclasts, and marrow with myelocytes. Stained with hematoxylin.

## PLATE 82.

FIG. 7. Original tumor. At the right of the picture there is cartilage, next to this osteoid tissue (nearly unstained), and to the left calcification of the osteoid tissue with marrow spaces advancing into the latter. Stained with hematoxylin.

FIG. 8. Hypertrophic cells of hyaline cartilage from a transplantation tumor (fowl 806, 1st generation). One cell has three nuclei. Stained with Mallory's chloride of iron hematoxylin.

## PLATE 83.

FIG. 9. From a transplantation tumor (fowl 815, 1st generation), showing transformation of collagenous connective tissue to cartilage. Note the large blood sinuses. Stained with hematoxylin.

FIG. 10. Another portion of the same growth, more highly magnified, showing collagenous neoplastic tissue (prechondral tissue) with cells resembling fibroblasts. Part of a blood sinus walled by endothelial cells is also shown. Stained with Mallory's chloride of iron hematoxylin.



FIG. 1.



FIG. 2.

(Tytler: A Transplantable New Growth of the Fowl.)



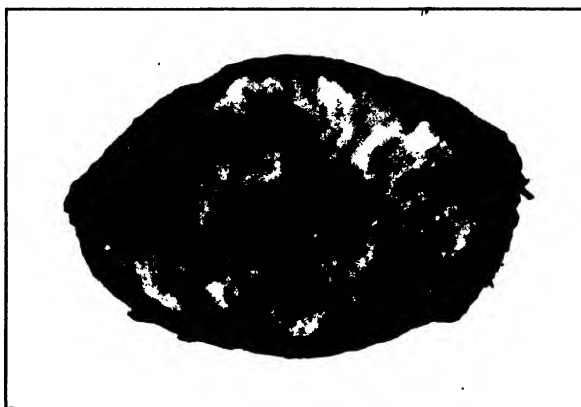


FIG. 3.

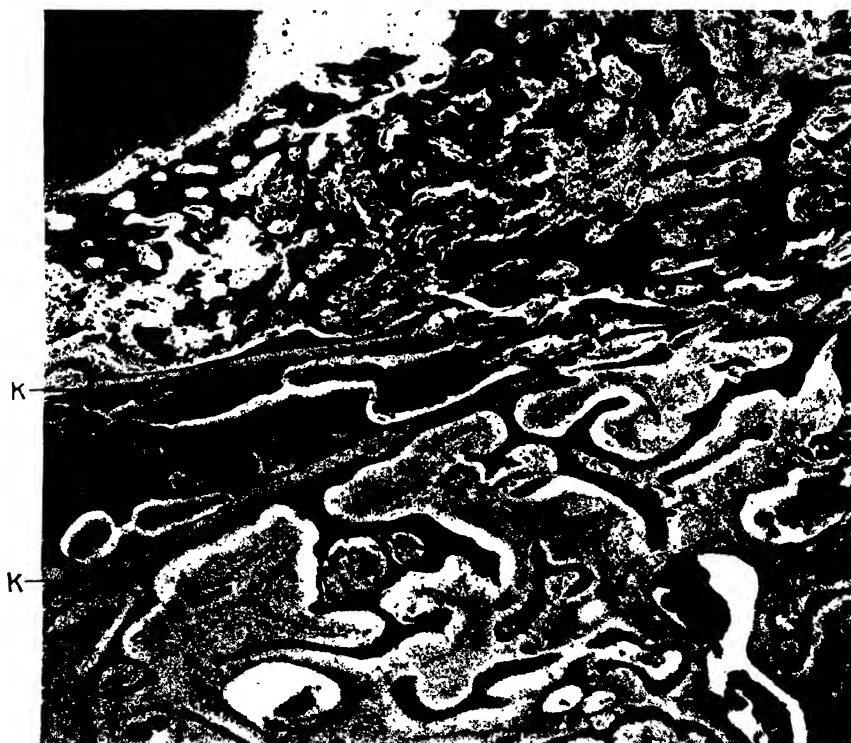


FIG. 4.

(Tytler: A Transplantable New Growth of the Fowl.)





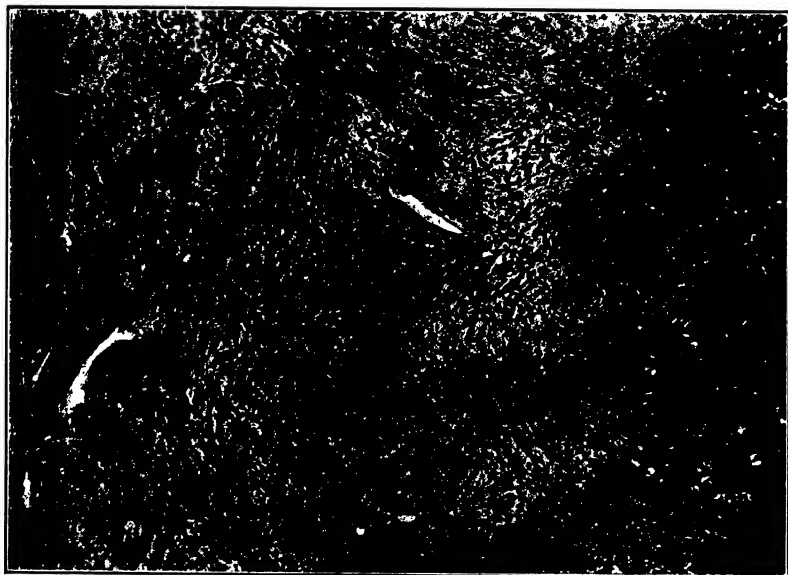


FIG. 5.

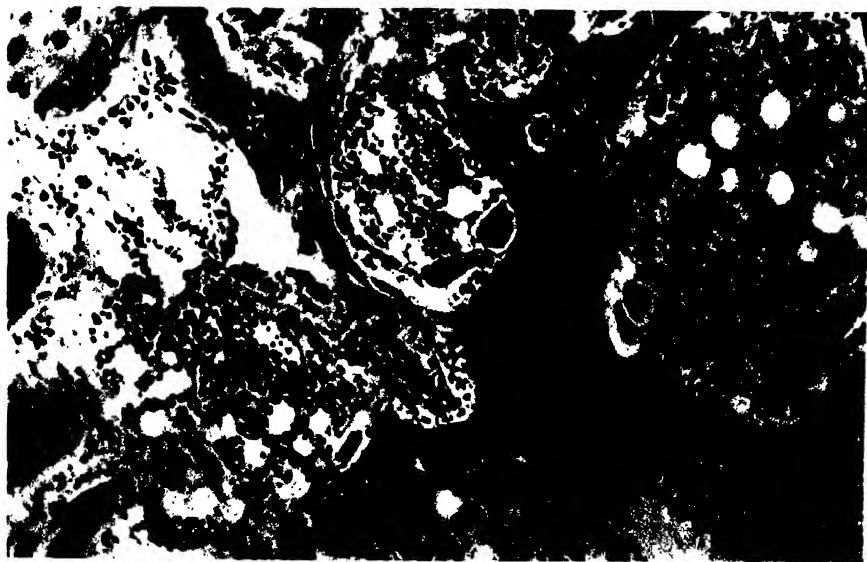


FIG. 6.

(Tytler: A Transplantable New Growth of the Fowl.)





FIG. 7.

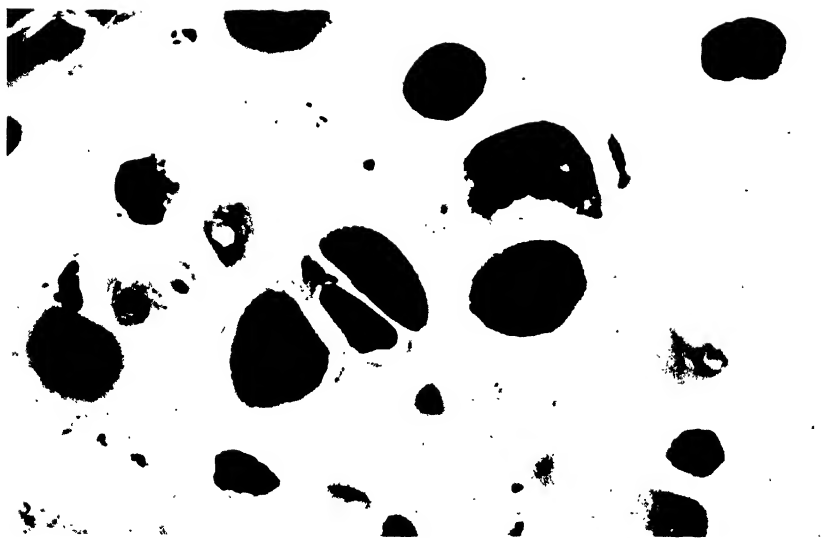


FIG. 8.

(Tytler : A Transplantable New Growth of the Fowl.)



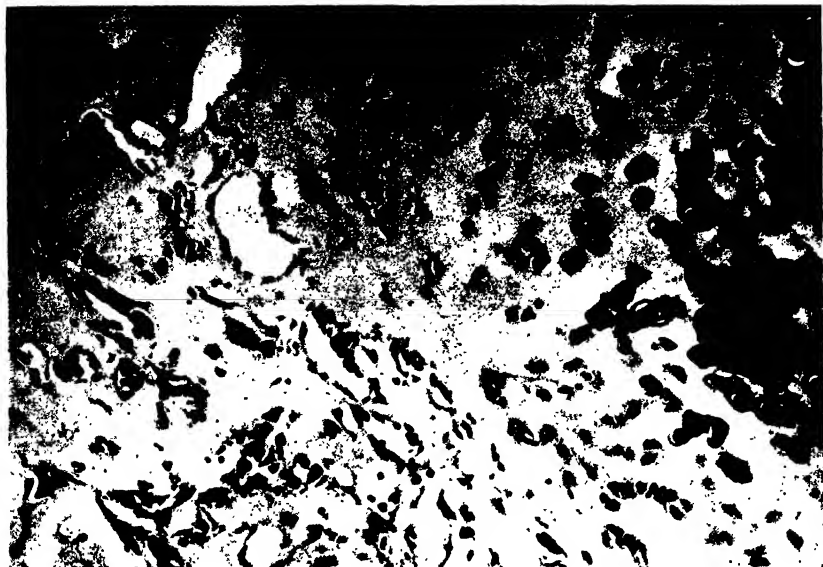


FIG. 10.

(Tytler : A Transplantable New Growth of the Fowl.)



## TRANSPLANTABILITY OF TISSUES TO THE EMBRYO OF FOREIGN SPECIES.

### ITS BEARING ON QUESTIONS OF TISSUE SPECIFICITY AND TUMOR IMMUNITY.\*

By JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

PLATES 84 to 89.

Gardeners and arboriculturists have known for centuries that plants of one species could be grafted on to another without in any way affecting the essential characters of either. In more recent years it has been shown that a like condition exists in certain of the lower animals. For example, Joest<sup>1</sup> working with worms found it possible to bring about a permanent union between *Lumbricus rubellus* and *Allolobophora terrestris*, each of the segments retaining its individual characteristics. Born,<sup>2</sup> Harrison,<sup>3</sup> and Morgan<sup>4</sup> have extended these observations to tadpoles. They have noted that even when the grafted portion is small and absolutely dependent on the major component for its circulation, nervous system, etc., it retains the characters of the original species from which it was derived. Formerly it was considered possible to graft certain tissues from lower animals<sup>5</sup> to human beings, but a more careful consideration and repetition of the experiments which suggested this have shown them to be erroneous.

In recent years much has been added to our knowledge of the laws underlying tissue grafting in warm blooded animals through the study of the transplantable tumors. These in general are governed by the same principles as those that govern the transplantation of

\* Received for publication, December 26, 1912.

<sup>1</sup> Joest, E., *Arch. f. Entwicklungsmechn. d. Organ.*, 1897, v, 419.

<sup>2</sup> Born, G., *Arch. f. Entwicklungsmechn. d. Organ.*, 1897, iv, 349.

<sup>3</sup> Harrison, R. G., *Arch. f. Entwicklungsmechn. d. Organ.*, 1898, vii, 430.

<sup>4</sup> Morgan, T. H., *Biol. Bull.*, 1899, i, 7.

<sup>5</sup> Allen, W., *Lancet*, 1884, ii, 875.



normal tissues. Not only do tissue grafts from warm blooded animals fail to survive when placed in a foreign species, but the barrier of tissue specificity also divides the different varieties of the same species. For example, certain tumors of the white mouse can not be successfully transplanted into grey, brown, or black mice of the same species, nor can certain tumors of the white rat be propagated in any other than white rats. Leo Loeb<sup>6</sup> has shown that the skin of a white guinea pig can be grafted on to the ear of a black guinea pig, but it eventually undergoes replacement with an inwandering of pigmented cells. A more recent and striking example of the strictness of the laws of specificity is that of a chicken sarcoma<sup>7</sup> which failed to grow in any other than blood-related animals during its first few transfers, later grew only in pure stock animals of the same variety, and would not grow in chickens of another variety until the malignancy had become greatly enhanced. This remarkable phenomenon of tissue specificity has been the subject of extensive investigation but its cause has not yet been determined. A general impression exists among experimenters that one of the immunity reactions is directed against the invasion of the foreign cells. The literature dealing with hemolysins and cytolsins need scarcely be gone into as the results reported offer no conclusive evidence. A theory of some interest, which explains the facts along other lines, is the athrepsia theory of Ehrlich,<sup>8</sup> which accounts for the failure of grafts in a foreign species by the lack of some specific food substance.

In the course of an investigation on the Rous chicken sarcoma in relation to chick embryo, it was observed that this tumor grew as well in the duck and pigeon embryo<sup>9</sup> (figure 1) as in that of the chicken, whereas it would not grow in the adults of these foreign species. This fact suggested to me the possibility that the chick embryo might serve as a suitable host for mammalian tissue as well. Inoculations of rat, mouse, and human tissue were made into the chick embryo and it was found that they grew as rapidly if not

<sup>6</sup>Loeb, L., *Arch. f. Entwicklungsmechn. d. Organ.*, 1898, vi, 1.

<sup>7</sup>Rous, P., *Jour. Exper. Med.*, 1911, xiii, 397.

<sup>8</sup>Ehrlich, P., *Arb. a. d. k. Inst. f. exper. Therap.*, 1906, No. 1, 84.

<sup>9</sup>Murphy, J. B., and Rous, P., *Jour. Exper. Med.*, 1912, xv, 119.

more so than in the adult of the native species<sup>10</sup> (figure 2). A typical experiment of this kind will be quoted.

*Experiment 1.*—A large rapidly growing Jensen sarcoma<sup>11</sup> of the rat was finely hashed and inoculated by means of a syringe and fine hypodermic needle into the outer membrane (fused chorion and allantois) of twelve chick embryos on the sixth day of incubation. On the eighteenth day of incubation the eggs were opened. Of the eight remaining alive, one showed no evidence of tumor; all the others showed a spherical mass projecting from the membrane at the point of inoculation. They varied in size from 0.1 to 1.6 cm. in their greatest diameter (figure 3). Histological examination showed these tumors to be made up of cells like those of the original tumor.

Bits of this tissue were inoculated into rats and there developed as a result tumors of the Jensen type.

Many experiments like the foregoing have been done. The growth of the Jensen tumor in the chick is always rapid. An accurate comparison of the rate of growth in the foreign embryo and the native adult would be of great interest, but this is rendered impossible on account of the nature of the injection site in the embryo. A certain proportion of the material injected into the egg escapes into the cavities and the amount clinging to the membrane and giving rise to the tumor varies on this account considerably. The results are sufficient, however, for the statement that the rate of growth is at least as rapid in the chick embryo as in the adult rat. It is of interest to note in this connection that while the normal temperature of the rat is 37.9°, the eggs were incubated at about 40° C.

#### DESCRIPTION OF RAT TUMORS IN THE CHICK EMBRYO.

For the work described in the present paper the Jensen sarcoma of the rat was used, a tumor widely known and the subject of much experimental work. Our strain gives a rapidly growing tumor in a high percentage of the animals inoculated. This tumor when introduced into the chick embryo grows readily where inoculated in the various membranes or in the body of the chick itself. For the general purposes of the experiment it has been found advantageous to use the outer membrane (fused allantois and chorion) for the reason that it lies just under the shell membrane and, therefore, can

<sup>10</sup> Murphy, J. B., *Jour. Am. Med. Assn.*, 1912, lix, 874.

<sup>11</sup> Jensen, C. O., *Ztschr. f. Krebsforsch.*, 1909, vii, 45.

be inoculated with a minimum amount of trauma. Furthermore, this membrane is the respiratory organ of the chick at this period and is rich in lymphatics and blood vessels. The inoculations were made between the fifth and seventh day of incubation and were allowed to grow till the eighteenth day.<sup>12</sup> Tumors resulting from such an inoculation of the Jensen sarcoma are found in the membrane as large globular masses lying in or suspended from the inner surface of the thin membrane by a broad pedicle. In the ten to twelve days of growth they sometimes attain the remarkable size of 2.1 cm. in diameter. The protruding masses are covered by a continuation of the chick membrane which gives them a smooth and glistening surface. Numerous dilated vessels are seen coursing through the membrane and penetrating the semitranslucent greyish tissue of the tumor itself. On section the nodules are found to be made up uniformly of this semitranslucent tissue, rarely with a small area of necrosis in the center but more frequently small areas of hemorrhage scattered here and there through the tissues.

Microscopically the cells making up these tumors resemble closely those of the same tumor in the rat; fairly large spindle cells with scant, deeply staining protoplasm and a large, clear vesicular nucleus with a nucleolus. They are so characteristic that there is no difficulty in distinguishing the tumor tissue from that of the chick even at the margin of the growth. The pattern of the tumor in the chick generally shows some variation from that in the rat (figures 4 and 5). In the latter the cells are seen in compact bundles coursing in various directions through the section, with a fair number of scattered, thin walled vessels. In the chick the arrangement of the cells is less compact, in some cases forming a loose network with clear spaces between, while in others the picture more closely approaches that seen in the native host. Whereas in the rat-grown tumors, mitotic figures are only occasionally seen, in those grown in the chick embryo they are found in almost every microscopic field, and as many as five mitotic figures have been seen in a single field of an oil immersion lens (figure 6). This finding is more striking when it is remembered that in the chick-grown tumors the number of cells

<sup>12</sup> Rous, P., and Murphy, J. B., *Jour. Am. Med. Assn.*, 1911, lvi, 741; Murphy, J. B., and Rous, P., *Jour. Exper. Med.*, *loc. cit.*

per field is much less than in the rat-grown ones. The vessels are much more numerous in the tumors of the embryo, here occurring in two forms, either ingrowths as clusters from the chick membrane (figure 7) or as individuals scattered throughout the tumor (figure 8). Apart from the thin continuation of the chick membrane which covers the tumor and the ingrowth of vessels with their scant accompanying stroma, there is no histological evidence of reaction on the part of the embryo to the invasion of foreign tissue. Certainly there are none of the sort attributed to a defensive reaction under similar conditions in the adult host.

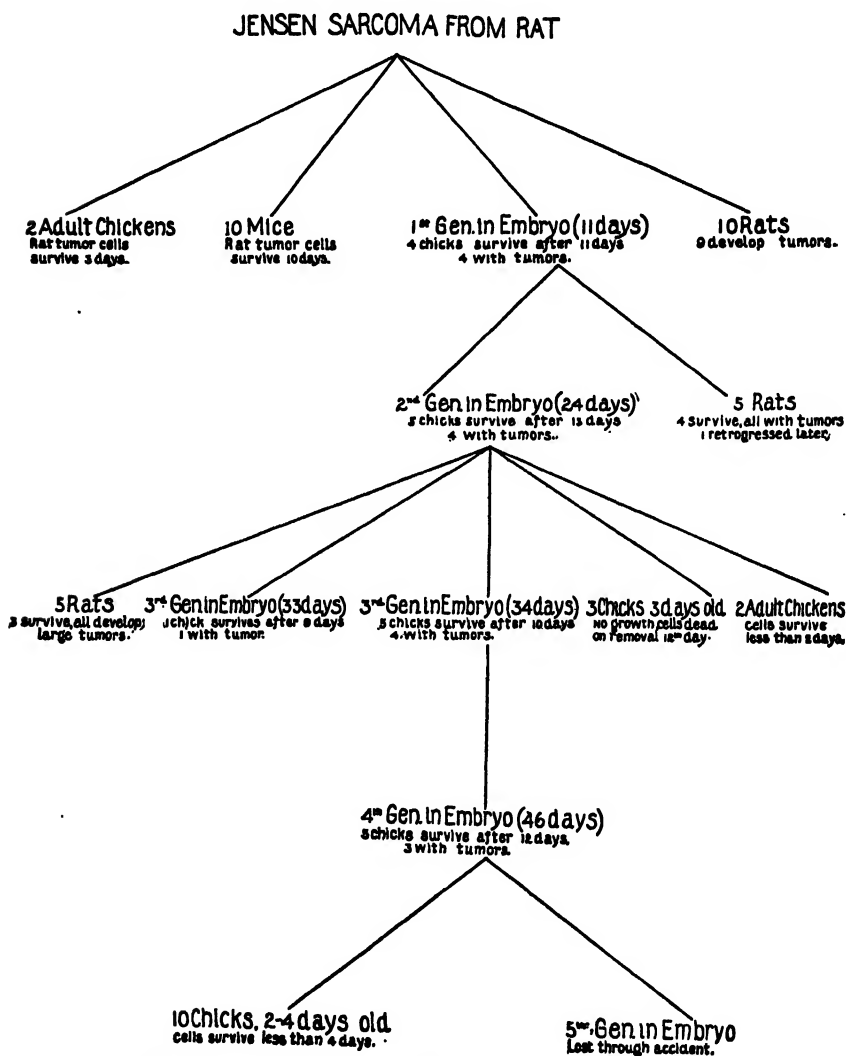
A microscopic study of the tumors occurring in the rat from inoculations with the chick-grown rat tumor shows them to be identical with those propagated in the usual way from rat to rat.

#### PROLONGED GROWTH OF RAT TISSUE IN CHICK EMBRYO.

The results of the experiments so far reported do not establish beyond question the utilization of the chick food material in the nourishment and growth of the rat cell, but they leave little doubt that this is the case. At the end of the twelfth day in the chick when the specific food carried with the graft from the native species must have been largely exhausted, there is no sign of lessened activity on the part of the tumor cells. Partly to settle this question beyond dispute, but more particularly to determine the changes, if any, that would be brought about in the rat cells by the prolonged growth in the embryos of a foreign species, the embryo-grown tumors were reinoculated into a second series of chick embryo. Not only did the tumors continue in rapid growth here, but they grew in turn when transplanted to a third or fourth series (figure 9). There seems to be no reason why by repeating transplantation growth cannot be prolonged indefinitely. Such an experiment with its controls will be quoted.

*Experiment 2.*—(Text-figure 1.) First generation. A rapidly growing Jensen rat sarcoma was finely hashed and injected into a series of seven day chick embryos. As control some of the same material was inoculated into the following animals: (1) ten rats, nine of which subsequently developed tumors; (2) ten mice, one of which was killed every forty-eight hours and whose tissues on microscopical examination showed survival of the rat cells till about the tenth day when they practically disappeared; (3) two adult

chickens, ten grafts in each, removed at twenty-four hour intervals, whose tissues showed survival of rat cells till the third day.<sup>23</sup> The eggs inoculated were opened on the eighteenth day of incubation, eleven days after the injection; four remained alive, all with large tumors.



**TEXT-FIG. 1.** This represents experiment 2 in outline.

<sup>23</sup> The criteria used here for survival were preservation of cell outline and the retention of the basic staining properties of the nucleus.

Second generation. The tumors from the above series of embryo were hashed and inoculated as follows: (1) into five rats, four of which survived and all developed tumors; and (2) into a second lot of embryos on the sixth day of incubation. These were opened on the nineteenth day (thirteen days later), five remaining alive, four with tumors

Third generation. The material from the last series was hashed and inoculated into the following animals: (1) five rats, three of which survived, all developing tumors; (2) three young chickens, hatched less than three days before, all developing small nodules which on removal twelve days later were found to be made up of reactive tissue with no rat cells present; (3) two adult chickens, ten grafts each, which on removal at forty-eight hour intervals showed no rat cell surviving even after the first forty-eight hours; and (4) into a lot of eight day embryos, five of which survived till the eighteenth day, four with tumors. The total number of days in the embryo was thirty-four.

Fourth generation. All the material from the above series was used to inoculate another lot of six day embryos. On the eighteenth day five chicks remained alive, three with tumors. Total number of days in embryo, forty-six.

Fifth generation. The material from the above was inoculated into: (1) ten newly hatched chicks about three days old, grafts from which were removed at forty-eight hour intervals beginning with the fourth day. None of the grafts showed surviving rat cells. It was also inoculated into (2) a lot of seven day embryos but these were unfortunately lost through an accident to the heat-regulating mechanism of the incubator.

These rat tumors of the second and third generation in the chick were similar in practically every respect to those grown in one chick for twelve days. The cells showed no morphological changes, but the tissue was perhaps more compact than in the early tumors.

This experiment, showing as it does the ability of the rat cells to survive and grow actively in the chick embryo for at least forty-six days, makes it certain that the rat cells utilize successfully the food offered by the blood of the foreign species. The question of adaptation therefore comes up for consideration. During this growth in the chick, have the rat cells acquired a real adaptation to the strange host? Apparently not. Morphologically there are no changes in the cell (figure 10). The tumors transferred from chick embryo to chick embryo grow neither better nor worse in the later periods than in the first chick to which they were transplanted. The material when returned to the rat caused a rapidly growing sarcoma which is always of the same type as the original tumor.

A comparative study of the fate of rat cells taken directly from the rat and those grown for a period in the embryo, when implanted in an adult, has proved of interest. Some of these results are men-

tioned in experiment 2, and numerous other experiments have been carried out along the same line. As previously mentioned the cells taken directly from the rat and inoculated into the adult chick will survive about three days. The period for the newly hatched chick is about the same. When, however, the rat cells have been grown for a period in the chick embryo and are then placed in the adult chicken, no evidence of them can be found after twenty-four hours. Practically the same results are obtained when newly hatched chickens are used as hosts. Instead of an adaptation to the new conditions the rat cells appear to have become less resistant during their growth in the chick embryos. All these findings show that the tumor cells have retained their essential characters despite the strange environment. Whether a longer dependence on a foreign species embryo would affect an adaptation remains to be seen, but certainly the present results offer little encouragement in this direction.

The conditions that make a chicken an unsuitable host for the growth of a foreign species cell seem, according to these experiments, to develop during the last two days of shell life, for it is certainly not present before that time and is present at the time of hatching. An attempt is being made now to establish the nature of this reaction and the exact time of its development.

#### GROWTH OF OTHER TISSUES IN THE CHICK EMBRYO.

Besides the rat sarcoma a variety of other tissues have been grown in the chick embryo for longer or shorter periods. Highly organized tissues of the chick itself, which fail to grow or grow poorly on transplantation to the adult, will grow in the embryo. Such tissues as kidney (figure 11), testicle, ovary, bone, cartilage, etc., have been grown for a period of from seven to ten days, but as yet no attempt has been made to carry them farther. Embryomata formed by inoculation of hashed chick embryo can be carried through several generations. They resemble the embryomata seen in the adult. After several transplantations of the embryomata the epithelial elements are less evident, the bone, cartilage, and connective tissue predominating.

Several other tissues of foreign species were grown with success,

among them the Ehrlich sarcoma and chondroma of the mouse, embryomata of the rat and mouse, a mammary carcinoma of the mouse (figure 12), and the Flexner-Jobling adenocarcinoma of the rat. Attempts with human tissue met with only moderate success, due perhaps to the time that elapsed between removal from the body and the introduction into the eggs. However, with this exception the tissue grew actively, with numerous mitotic figures and a copious blood supply from the embryo.

#### DISCUSSION.

The question of an adaptation of the rat cells to a new host is naturally of great interest. The evidence of change in the tissue is slight, if present at all. Instead of becoming capable of a longer survival in the adult or even newly born individual of the foreign species after the long dependence on its embryo, the rat cells have become more susceptible to the unfavorable conditions existing in these hosts. As mentioned above, this retention of characteristics has an analogy in the plants and lower animal forms. The branches of one species of tree may be grafted into the trunk of another, but though it depends upon the sap supplied by the roots of a foreign species, it retains all its own characteristics. In the lower animals where it is easy to graft parts of closely related species, it is observed that where small pieces of one species can be grafted on to another, the minor component will retain its characteristics. When a part of this minor component is removed, the regeneration that takes place will be like the minor component, although dependent on the major component of a foreign species for its nervous system, alimentary tract, and vascular system.

The fact that rat cells will grow in the chick embryo almost until the time of hatching, and when transferred to second, third, and fourth lots are found at the end of forty-six days of continuous growth in the foreign species embryo to be growing as actively as the same tissue in the rat, leaves no possibility of doubt that the avian food is just as suitable as the native food for the maintenance and growth of the tissue. The inability of the embryo to elaborate a defensive substance against this invasion is not surprising when it is remembered that in very young animals certain of the immunity



substances, as hemolysins, can only be developed to a very slight extent or not at all.<sup>14</sup>

With the evidence at hand from these experiments, it would seem that the athrepsia theory of Ehrlich does not apply to this special case. We have evidence of the ability of the rat cells to utilize nutritive substances from the embryo of a foreign species. It might be suggested that the embryo offers a food of less specific composition and therefore utilizable by a foreign species cell. The evidence presented by the experiments in adults would favor another interpretation. If the temporary survival and growth of the cells when transferred to an adult of a foreign species is to be accounted for by the specific food carried with the graft from the native animal, we should expect the tissues to live an equal length of time whether in a closely related or more distantly related species. But such is not the case. I have shown here that while the rat cells can live for from nine to eleven days in the mouse, they die off in the adult chicken by the third day. It would seem much more likely that there is a defensive mechanism whose strength and rapidity of reaction depend upon the degree of relationship, being more prompt and violent the more foreign the tissue introduced. We have at least one example in immunity reactions of the fine gradation between species, namely, in the precipitin phenomena.<sup>15</sup> Here a precipitin developed for the sera of one animal will give a heavy precipitate with all sera from animals of the same species and a progressively fainter reaction with sera of other species, depending on the distance of relationship.

The type of reaction that prevents the growth of a tissue in a foreign species adult is as yet a matter of speculation. That it is a property which is developed rather than one naturally present in the adult is strongly suggested by the experiments of Russell.<sup>16</sup> He found that the cells of a mouse survived and multiplied in a normal rat for more than nine days, while in a rat previously immunized the

<sup>14</sup> Famulener, L. W., *Collected Studies from the Research Laboratory, Department of Health, New York*, 1911, vi, 199.

<sup>15</sup> For the literature and a comprehensive review of the subject, see Nuttall, G. H., *Blood Immunity and Blood Relationship*, Cambridge, 1904.

<sup>16</sup> Russell, B. R. G., *Third Scientific Report of the Imperial Cancer Research Fund*, 1908, 341.

graft was rapidly disintegrated, all cell outlines being obliterated by the fourth day. Lambert and Hanes<sup>17</sup> have added the observation that rat and mouse tissues will grow almost as well in plasma from an alien as from the native species. They have further shown<sup>18</sup> that if the animal from which plasma is obtained is previously immunized with the living cells of the foreign species, the plasma will inhibit or actually prevent any such activity. It has been suggested that this may be due to a cytotoxin. We have little conclusive evidence either from these experiments of Lambert and Hanes or from mine as to the exact nature of this defensive mechanism. Little more can be said at present than that such a defense exists.

#### SUMMARY.

Inoculation of the Jensen rat sarcoma into the developing chick embryo gives a rapidly growing tumor at the site of inoculation, whether in the membranes or in the body of the chick itself. These tumors by transfer from embryo to embryo can be kept going for as long as forty-six days, and perhaps indefinitely in the foreign species. The rat cells show no morphological change even after a very long dependence. Their biological characters are also retained, as is shown by the fact that the cells when replanted in the rat, after a prolonged sojourn in the chick, will produce a rapidly growing sarcoma of the Jensen type. These rat tissues grown for long periods in the chick show no adaptation to the new species, being destroyed even more rapidly when placed in the adult chicken than cells taken directly from the rat. Morphologically the cells retain a close resemblance to those in the original tumor.

Other tissues grown in chick embryo are various embryonic cells from the chicken, mouse, and rat, the Ehrlich sarcoma and chondroma of the mouse, a mammary carcinoma of the mouse, the Flexner-Jobling adenocarcinoma of the rat, and a human sarcoma.

#### EXPLANATION OF PLATES.

##### PLATE 84.

FIG. 1. A chicken sarcoma growing in the yolk sac of a pigeon embryo (A), seven days after inoculation.

<sup>17</sup> Lambert, R. A., and Hanes, F. M., *Jour. Exper. Med.*, 1911, xiv, 129.

<sup>18</sup> Lambert, R. A., and Hanes, F. M., *loc. cit.*, p. 453.

FIG. 2. The Jensen rat sarcoma in a chick embryo. This animal was killed on the eighteenth day of incubation, seven days after inoculation. The tumor lay partly in the cranial cavity, extending through an opening in the skull and protruding outward.

PLATE 85.

FIG. 3. The Jensen rat sarcoma in the outer membrane of an eighteen day embryo, eleven days after inoculation. The tumor measured 1.7 by 1.5 cm.

FIG. 4. A section of rat sarcoma after twelve day's growth in a chick embryo. *A* and *B* = mitotic figures.

PLATE 86.

FIG. 5. The Jensen rat sarcoma as it appears growing in the rat.

FIG. 6. A rat sarcoma after eleven days in a chick embryo, showing five mitotic figures.

PLATE 87.

FIG. 7. A tuft of vessels from the chick membrane growing into a rat tumor which it supports. The membrane is on the right side.

FIG. 8. A second and more common type of vessel distribution in a rat tumor growing in chick embryos. The rat cells are seen clustered around the small scattered vessels. *A* = mitotic figures.

PLATE 88.

FIG. 9. A group of rat sarcomata in a chick membrane, second generation in the embryo, with a total of nineteen days of continuous growth in the foreign species. The central tumor measured 1.4 by 1.4 cm.

FIG. 10. A section of rat sarcoma after four generations in chick embryos (forty-six days). *A* = mitotic figures.

PLATE 89.

FIG. 11. Growing kidney tubules in the outer membrane of an eighteen day chick, resulting from the inoculation of embryonic kidney.

FIG. 12. A mammary carcinoma from a mouse growing in a chick embryo. The epithelial cells are seen in clusters.



FIG. 1.



FIG. 2.

(Murphy : Transplantation of Tissue to Embryo.)





FIG. 3.



FIG. 4.

(Murphy : Transplantation of Tissue to Embryo.)





FIG. 5.

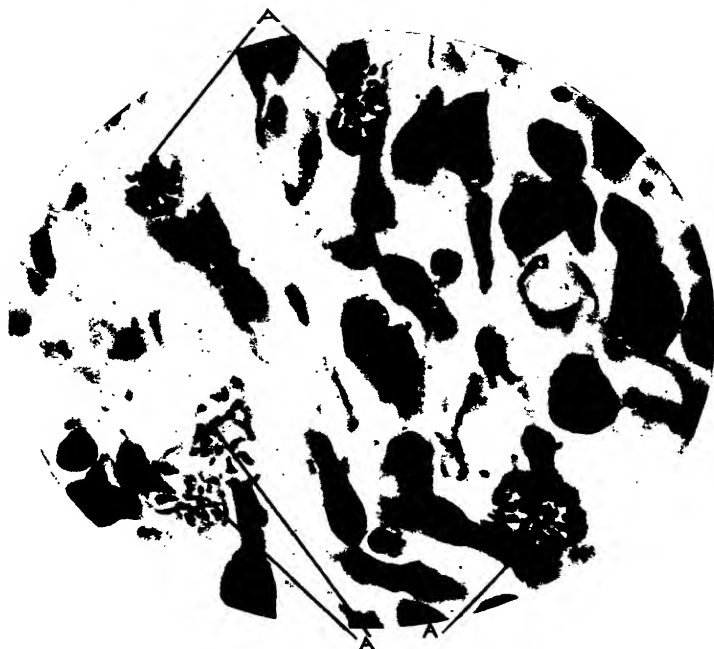


FIG. 6.

(Murphy : Transplantation of Tissue to Embryo.)





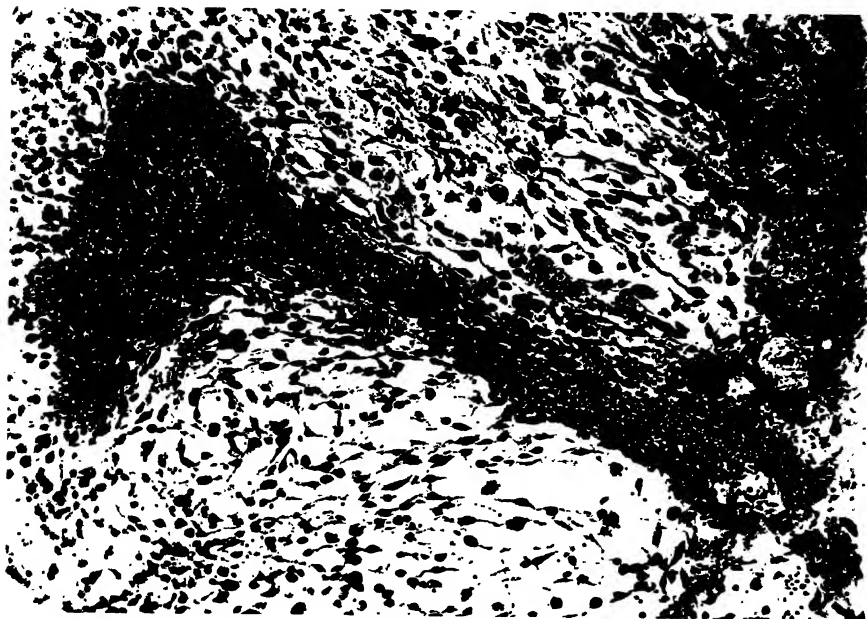


FIG. 7.

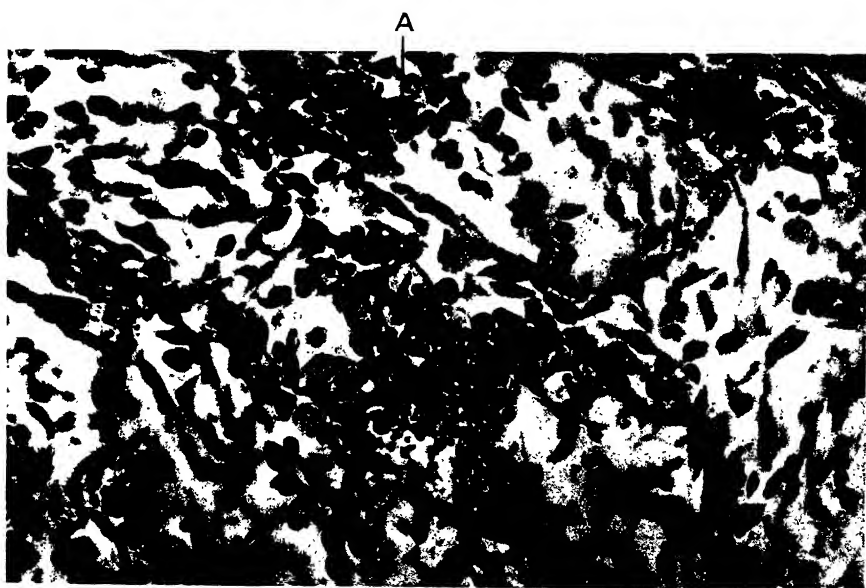


FIG. 8.

(Murphy : Transplantation of Tissue to Embryo.)



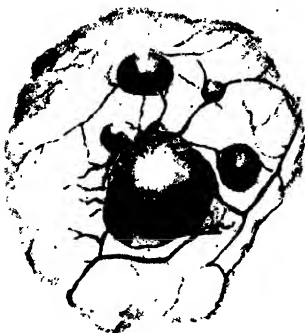


FIG. 9.

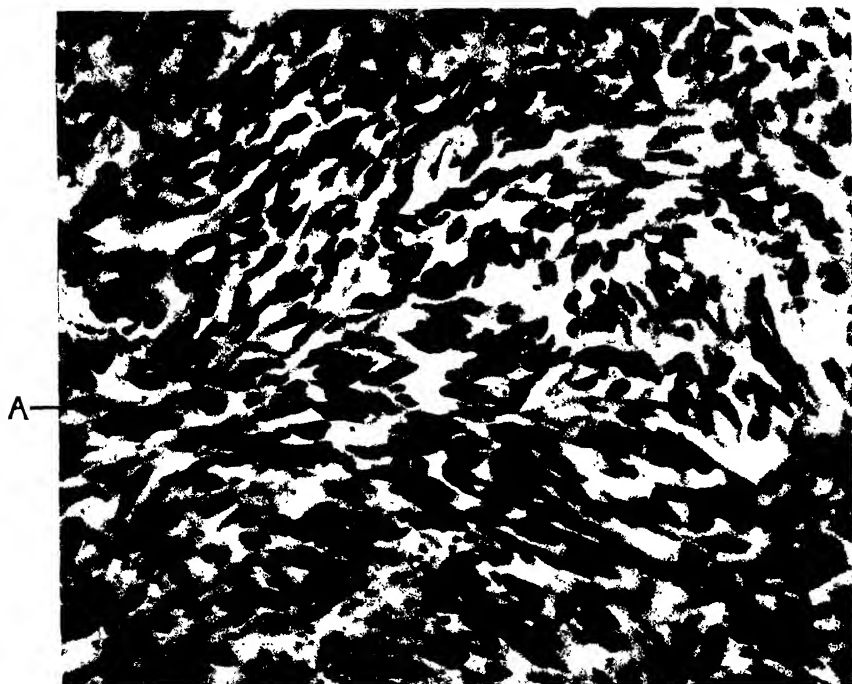


FIG. 10.

(Murphy : Transplantation of Tissue to Embryo.)



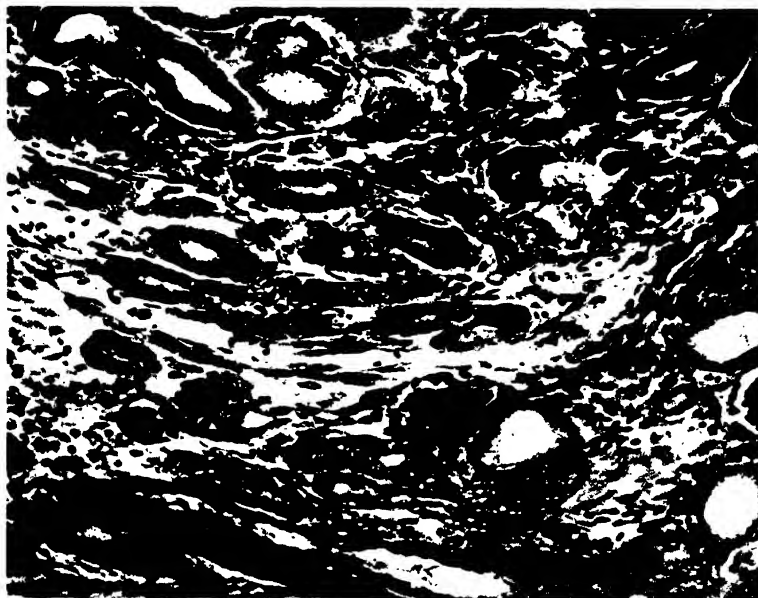


FIG. 11.

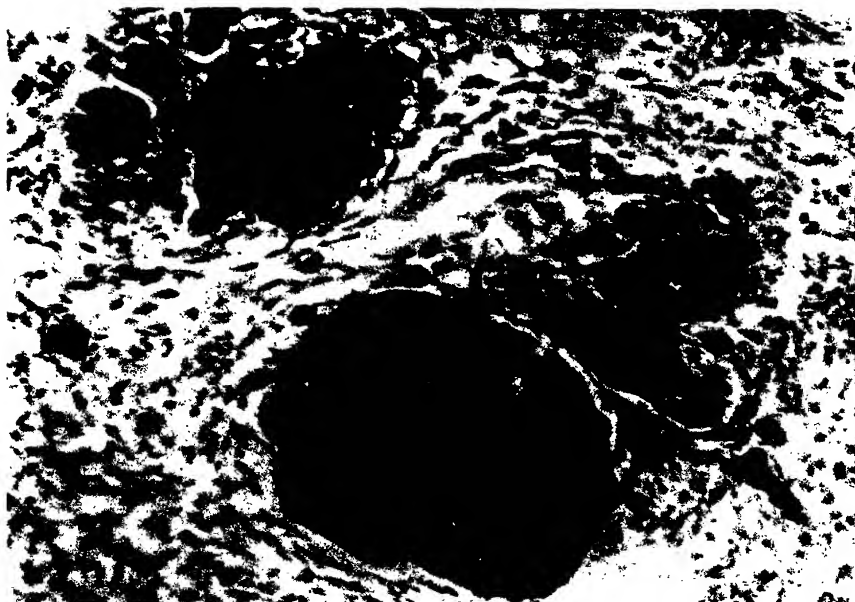


FIG. 12.

(Murphy : Transplantation of Tissue to Embryo.)



## FALSE TRANSITIONS BETWEEN NORMAL AND CANCEROUS EPITHELIUM.\*

By PEYTON ROUS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

PLATES 90 TO 97.

The question as to whether cancer arises directly from the normal structures amid which it first appears has considerable theoretical importance and has been one of the most debated in pathology. Yet despite much recent investigation it has not been entirely decided. Some workers report that the apparent transitions between normal and cancerous epithelium so often found are mere appositions, not transitions in the true sense of the term. In this conclusion they find a new argument for the theories which involve an independent origin for cancer. Other workers again describe what appear to be most convincing transition stages; and certainly pictures showing such transitions are very familiar to the consulting pathologist. For the purpose of the present communication the literature scarcely need be reviewed. It is not our aim to dispute the existence of true transition stages, or, on the other hand, to affirm it, but to demonstrate that the histological appearances of transition can result from a secondary union of normal and cancerous epithelium, and probably do so result frequently.

Several years ago while examining into the fate of implanted mixtures of embryo and tumor, the author noted in several instances a direct union of carcinoma with the stratified squamous epithelium of the embryo.<sup>1</sup> This came about when the normal and cancerous epithelium, growing along a surface, met end to end. The finding suggested an experiment, as follows:

*Experiment.*—Eight normal rats and seven carrying the Flexner-Jobling adenocarcinoma were used. The tumor employed was the adenocarcinoma just

\* Received for publication, January 10, 1913.

<sup>1</sup> Rous, P., *Jour. Am. Med. Assn.*, 1910, liv, 1939; *Jour. Exper. Med.*, 1911, xiii, 239.



mentioned, a growth which had its primary origin in the seminal vesicle. Under ether a disc of skin was cut from each side of the normal rats and on the exposed surfaces (which did not bleed) there were spread with the flat of a knife a few fragments of finely ground tumor tissue. The dressing was a flexible paraffin mixture which became fluid at 47° C. Of itself this mixture does not induce atypical epithelial proliferation. In order to rule out a possible damage to the tumor tissue from heat the dressing was put on the wound as a thin, solid sheet warmed to flexibility. It was kept in place with gauze and adhesive plaster.

In some instances a second dressing was made on the sixth day, in others the first one remained undisturbed until the rats were killed and examined at the ninth to eleventh day. In only one rat did any infection develop. In all the epithelium rapidly regenerated and in several it had covered the wound surface by the ninth day. On twelve of the sixteen disc-shaped wounds a tumor developed as one or more discrete, whitish nodules lying for the most part below the surface and sometimes extending beyond the area denuded. In some instances the thin edge of epithelium advancing to cover the wound appeared intimately attached to the pale, exposed tumor. The specimens were cut into blocks vertical to the surface and serial sections were made of them.

In the case of the rats which already carried the Flexner-Jobling tumor at the time of operation the tumor tissue was exposed by slicing off the skin over it and a portion of the capsule. Infection was frequent and the exposed tumor usually became necrotic. In only two instances did the conditions appear favorable to a junction of the normal and carcinomatous epithelium. In one of these cases such a junction was found microscopically.

The results of the experiment are best shown by the photographs (figures 1 to 8). Even macroscopically there often appeared to be some connection between the thin film of regenerating epithelium and the exposed carcinoma. Microscopically a direct union of the two was not only frequent, but it many times took the form of a gradual histological transition between the stratified, squamous epithelium and the adenocarcinoma. That these are actual transitions involving a malignant transformation of the regenerating epithelium is scarcely possible. Previous knowledge is entirely against it; and, furthermore, the transition is always a transition to the peculiar type of adenocarcinoma that was inoculated,—a growth with cells much larger than those of the resting or regenerating squamous epithelium, containing a large and more deeply staining nucleus. The adenocarcinoma, when propagated alone, has shown no tendency to the squamous form.

The transition pictures are found under two conditions. The regenerating epidermis may not yet have extended over the carcinomatous surface and has united at its advancing edge with the

neoplastic epithelium (figures 1, 2, and 4); or the epidermis has more or less completely covered the tumor, and prolongations from it into the deeper tissue connect directly with strands or acini of the carcinoma (figures 3 and 5). Probably in the latter instance there was originally an end-to-end meeting on or near the surface, but the regenerating epidermis spreading beyond this junction has placed it underneath.

In addition to transition stages there have been found many meeting points of skin and cancer without any union of the two (figure 4), and many in which a union occurred but a definite line of demarcation between the two remained (figures 6 and 7). Occasionally individual carcinoma cells appear to have been caught up in the regenerating epithelium and, proliferating subsequently, to have extended out from it (figure 8). This finding is of interest in connection with the doctrine of "embryonic rests." If such rests exist in normal epithelium, their proliferation might very well result in such pictures, or in transition pictures that would completely conceal the real origin of the tumor.

There seems to be no doubt that, if a squamous-celled carcinoma had been available for the experiment, even more deceptive transition pictures than the ones figured would have been obtained. Each small ulceration of an epithelioma of man gives opportunities similar to those in the experiment for a secondary union of the cancer with the normal skin and thus for the development of transition pictures. The findings detailed in the present paper should make for greater caution in the interpretation of the histological appearances of transition between normal and cancerous epithelium.

#### EXPLANATION OF PLATES.

##### PLATE 90.

FIG. 1. End-to-end union of the regenerating epidermis and the adenocarcinoma. *sk* = epidermis; *c* = carcinoma; *sq* = epidermal pearl.

At *T* there is a histological transition between the carcinoma and the epidermis.

##### PLATE 91.

FIG. 2. To be viewed in connection with figure 3. End-to-end union of the regenerating epidermis and the adenocarcinoma.

## PLATE 92.

FIG. 3. Another section of the specimen shown in the preceding figure. The connection between the squamous epithelium here found deep in the tissue and the surface epithelium is made plain by figure 2. *sk* = regenerating epidermis; *c* = carcinoma; *sq* = squamous epithelium of epidermal prolongations into the deeper tissue; *T* = union between the carcinoma and the squamous epithelium, with transition forms.

## PLATE 93.

FIG. 4. Union of the carcinoma and the basal layer of cells of the regenerating epidermis. The histological transition between the epidermal cells and those of the cancer acinus is well shown. At the surface of the wound there is a meeting between the epidermis and carcinoma but no union.

## PLATE 94.

FIG. 5. Union of individual carcinoma cells with those of a downward prolongation of the regenerated epidermis. At the upper border of the figure is a portion of the surface layer of epidermis. Its connection with the prolongation at the center of the picture is not shown. *t* = tumor cells united with the prolongation; *c* = free tumor cells.

## PLATE 95.

FIG. 6. Juxtaposition of carcinoma cells and those of the regenerating epithelium, without transition forms. *c* = carcinoma; *sk* = epidermis.

## PLATE 96.

FIG. 7. Juxtaposition of carcinoma cells and those of a prolongation of the regenerating epithelium. No transition forms. *c* = carcinoma.

## PLATE 97.

FIG. 8. An island of carcinoma cells (*c*) lying within the regenerated epidermis. Numerous carcinoma cells are scattered in the deeper tissue.



FIG. 1.

(Rous : Transitions between Epithelium.)





FIG. 2.

(Rous: Transitions between Epithelium.)





FIG. 3.

(Rous: Transitions between Epithelium.)





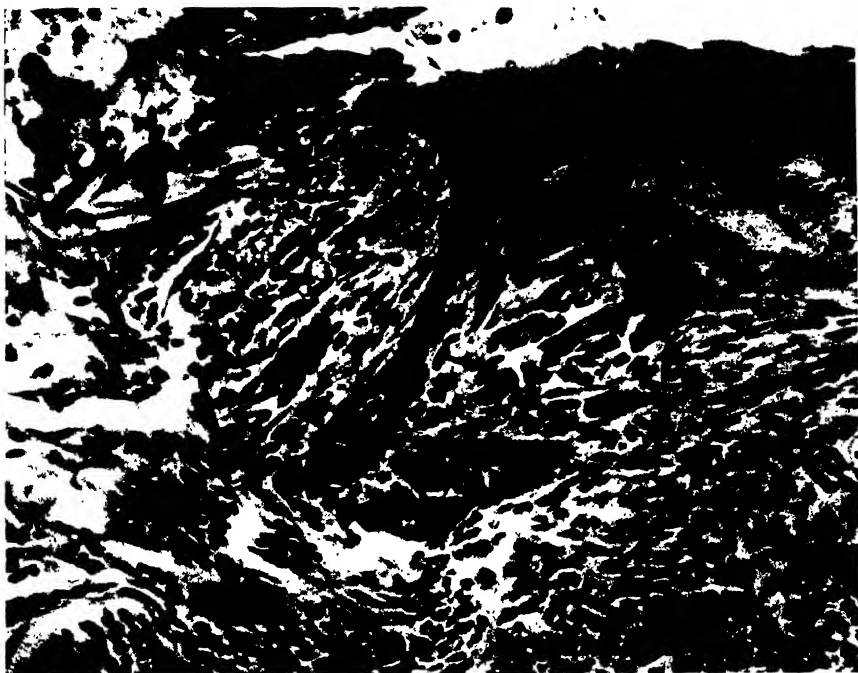


FIG. 4.

(Rous : Transitions between Epithelium.)





FIG. 5.

(Rous: Transitions between Epithelium.)





FIG. 6.

(Rous: Transitions between Epithelium.)



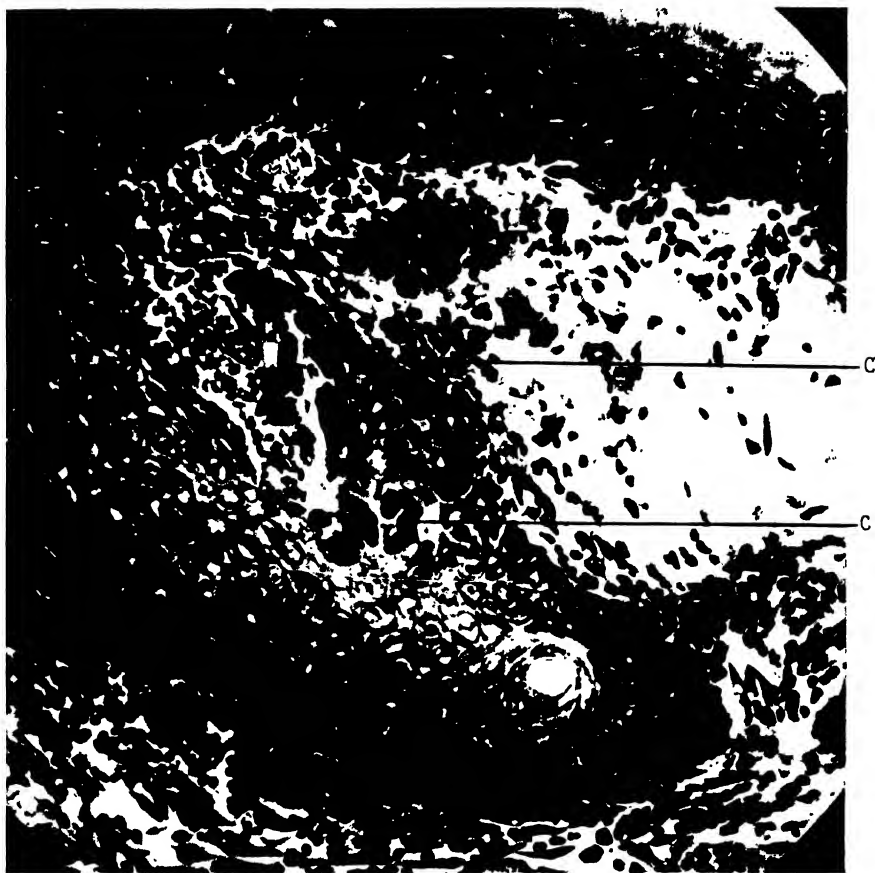


FIG. 7.

(Rous : Transitions between Epithelium.)







FIG. 8.

(Rous : Transitions between Epithelium.)



## STUDIEN ÜBER DEN NACHWEIS DER SPIROCHAETA PALLIDA IM ZENTRALNERVENSYSTEM BEI DER PROGRESSIVEN PARALYSE UND BEI TABES DORSALIS.

VON PROF. DR. H. NOGUCHI.

*(Aus den Laboratorien des Rockefeller Institute for Medical Research,  
New York.)*

Es wird heutzutage wohl von den meisten Klinikern anerkannt, dass die progressive Paralyse sowohl wie die Tabes dorsalis ätiologisch mit einer früher stattgehabten syphilitischen Infektion in Zusammenhang gebracht werden müssen. Eine grosse Anzahl, auf schärfster Beobachtung und gründlichster Systematik beruhender, Studien zahlreicher Forscher, insbesondere die Arbeiten von Esmarch und Jessen, Kjelberg, Fournier, Erb, Winge und Sandberg, Stenberg, Althaus, Virchow, Krafft-Ebing, Kraepelin, Gowers, Möbius, Strümpell, Raymonds, Binswanger, Leredde, Hallopeau, Homén, Mendel, Nonne, Mott und Collins stellen die Existenz einer unleugbaren Beziehung zwischen der syphilitischen Infektion einerseits und den unter dem Sammelbegriff „Parasyphilis des Zentralnervensystems“ bekannten Krankheitsbildern andererseits aufs deutlichste fest. Die Behauptung, dass die „meta- oder parasyphilitischen Krankheiten“ mit einer früherenluetischen Infektion zusammenhängen, gründet sich hauptsächlich auf die Tatsache, dass die Mehrzahl der an allgemeiner progressiver Paralyse oder an Tabes dorsalis erkrankten Individuen nachweislich in einer früheren Periode ihres Lebens Syphilis akquiriert hatte. Da nun allerdings die Tabes und die progressive Syphilis die der Syphilis anderer Organe eigentümlichsten histologischen Bilder im Zentralnervensystem vermissen lassen, ausserdem auch antiluetische Therapie diese Erkrankungen nicht beeinflusst, ist die oben erwähnte Beziehung zur Lues vielfach angezweifelt

worden: und in der Tat, gäbe es nicht das Krankheitsbild der typischen Zerebrospinalmeningitis mit wohlcharakterisierten histologischen Läsionen, leicht beeinflussbar durch methodische anti-luetische Therapie, so möchte man fast zu der Annahme neigen, dass Gehirnlues und spinale Syphilis besondere in anderen Organen nicht auffindbare Eigentümlichkeiten aufweisen. Da es nun aber viele Krankheitsfälle gibt, welche sich histologisch und in spezifisch-therapeutischer Hinsicht als typische Fälle von zerebraler und zerebrospinaler Syphilis darstellen, wurde es natürlich schwierig, die progressive Paralyse und die Tabes unter den engeren Begriff wirklich syphilitischer Erkrankungen einzureihen. Fournier und andere verfielen auf den Ausweg, den Begriff der Parasyphilis oder Metasyphilis einzuführen, während andere Tabes und Paralyse als postsyphilitische Erkrankungen bezeichnet wissen wollten. Einige Forscher bekannten sich zu der Annahme, dass die der Paralyse und Tabes eigentümlichen Läsionen auf den schädigenden Einfluss gewisser toxischer Substanzen zurückzuführen seien, welche sich infolge einer durch die frühere syphilitische Infektion ausgelösten Stoffwechselanomalie von unbekannter Qualität bilden sollten. Man hätte es demnach mit einer Nachwirkung auf der Basis einer früheren Infektion, nicht mit dem Infektionserreger selbst zu tun, weshalb man sich auch von einer gegen den Infektionserreger selbst gerichteten Behandlung keine günstigen Resultate versprechen konnte.

Die Frage indessen, ob wir die sogenannten para- oder post-syphilitischen Erkrankungen des Zentralnervensystems auf das noch lebende Virus selbst beziehen sollen oder ob wir dieselben lediglich als postinfektiöse Nachkrankheiten, als solche nicht mehr in unmittelbarer Beziehung zum infektiösen Virus stehend, ansehen sollen, diese Frage ist nicht nur theoretisch äusserst interessant, sie ist auch praktisch von einschneidender Wichtigkeit, da ja unser therapeutisches Handeln durchaus von ihrer Beantwortung abhängen wird. Seit der epochemachenden Entdeckung der *Spirochaeta pallida* in allen Stadien der Syphilis durch Schaudinn und Hoffmann haben viele hervorragende Pathologen wiederholt versucht, die Syphilisorganismen im Gewebe des erkrankten Zentralnervensystems in Fällen der sogen. parasyphilitischen Affektionen

nachzuweisen, aber alle ohne Erfolg. Dieser Misserfolg bewies jedoch keineswegs die Abwesenheit der Pallida, vielmehr machten es die Befunde von positiver Wassermann-Neisser-Bruckscher Reaktion im Blut und in der Zerebrospinalflüssigkeit, die Pleozytose und Erhöhung des Globulingehaltes der letzteren, sowie das stete Fortschreiten der Erkrankungssymptome sehr wahrscheinlich, dass das infektiöse Virus in den erkrankten Organen dennoch gegenwärtig sei. Ausserdem kam eine grössere Anzahl Fälle von inkompleter Tabes dorsalis zur Beobachtung, in denen zweckmässig eingeleitete und gründlich durchgeführte Salvarsan- oder Neosalvarsankur die Erkrankungen subjectiv und objektiv nachweisbar günstig beeinflusste<sup>1)</sup>.

Mein Interesse an diesen die „Parasyphilis“ betreffenden Fragen wurde rege, als ich mich experimentell eingehender mit der Erforschung der Syphilis befasste, speziell aber dann, als meine Aufmerksamkeit durch ein eigenartiges Phänomen gefesselt wurde, welches ich bei meinen Studien der kulturellen Eigenschaften der *Spirochaete pallida* beobachtete. Ich konnte mich nämlich bei der Beobachtung meiner Pallidakulturen davon überzeugen, dass unter gewissen Bedingungen die *Spirochaete pallida* in Reinkultur zahlreiche kleinste Körner bildet, und dass von diesen Körnern nach Uebertragung in einen passiven Nährboden wieder Spiralformen aussprossen. Diese Wahrnehmung schien mir auf die Möglichkeit hinzuweisen, dass in Fällen von Parasyphilis die *Spirochaete pallida* vielleicht in Körnchenform vorhanden sei, und dass gerade die Abwesenheit der gewöhnlichen Pallidaspiralformen in den Gehirnen an progressiver Paralyse gestorbener Patienten es verschuldet haben möge, dass so viele ausgezeichnete Beobachter negative Resultate hatten. Unter diesem Eindruck machte ich mich daran, zahlreiche Präparate von paralytischen Gehirnen zu untersuchen. Wie ich und Dr. Moore<sup>2)</sup> anderweit schon berichtet haben, konnte ich binnen kurzem die typischen Spiralformen der Pallida in 12 von 70 Fällen meiner ersten Serie nachweisen. Dies ganz unerwartete Ergebnis

<sup>1)</sup> Swift and Ellis: The direct treatment of syphilitic diseases of the central nervous system. New York Medical Journal, July 1912.

<sup>2)</sup> Noguchi and Moore: A demonstration of *Treponema pallidum* in the brain in cases of general paralysis. Journ. Exper. Med., Bd. XVII, No. 2, Februar 1913. (Ref. d. W. No. 8, S. 446.)

erregte mein Verlangen, noch weitere Fälle zu sammeln, um womöglich Aufklärung darüber zu erhalten, warum die Organismen nicht vorher schon nachgewiesen worden waren. Es ist allerdings wahr, dass das Auffinden der Pallida in vielen Präparaten unsäglich langwierig, ja oft unmöglich ist. Ich will im Nachstehenden nun meine Erfahrungen über den Gegenstand mitteilen, und will insbesondere einige technische Details erörtern, die mir, wie ich glaube, das Auffinden der Pallida im Zentralnervensystem von Fällen der progressiven Paralyse und der Tabes dorsalis erleichterten.

Bisher habe ich 200 Gehirne von Fällen der allgemeinen Paralyse und 12 Rückenmarke von Fällen von Tabes dorsalis untersucht. Den Herren Dr. Cotton, Dr. Dunlap, Dr. Elser, Prof. Ewing, Dr. Fischer, Prof. Graves, Dr. Hough, Dr. Kaplan, Dr. Lambert, Dr. Moore, Captain Nichols, Dr. Orton, Dr. Rosanoff, Dr. Smith, Prof. Spiller, Prof. Weisenburg und Dr. Zablicki möchte ich an dieser Stelle für das mir so freundlich zur Verfügung gestellte Material meinen ergebensten Dank aussprechen.

Die Präparate wurden nach der von Levaditi angegebenen Färbemethode mit einigen noch später zu besprechenden Modifikationen angefertigt. Immerhin mag hier gleich gesagt werden, dass die ältere Methode von Levaditi in einigen Fällen ganz gute Resultate gegeben hat.

In der Gesamtzahl der untersuchten Fälle (200 paralytische Gehirne) konnte die *Spirochaeta pallida* 48 mal gefunden werden. Das Alter der Patienten, von denen diese durch positiven Pallidabefund ausgezeichneten Gehirne stammten, schwankte zwischen 29 bis zu 75 Jahren, und selbst in einem Falle, wo die Erkrankung nachweislich 6 Jahre gedauert hatte, gelang es, die Spirochäten nachzuweisen. In einem der mir von Dr. Lambert und Dr. Rosanoff zur Verfügung gestellten frischen Paralytikergehirnen gelang es, die Pallida im Dunkelfeld nachzuweisen, doch war ihre Zahl äusserst klein, und in den gefärbten Präparaten habe ich sie in diesem Falle dann nicht wieder nachweisen können. Sehr schwierig ist andererseits die Untersuchung des Rückenmarkes. Im Beginn meiner Untersuchungen machte ich Querschnitte vom Rückenmark, doch erschwerte das strukturelle Gefüge des Organs — eine Unzahl

feinster Nervenquerschnitte und von Neurogliafasern — die Uebersicht ganz enorm. Zum Teil konnte ich diese Schwierigkeit überwinden, indem ich Längsschnitte vom Rückenmark anfertigte, und in einem der 12 Fälle ist es mir dann geglückt, im Hinterstrang des Dorsalmarkes die Gegenwart der Spirochäten festzustellen. Sie waren an Zahl sehr gering, und ihre Auffindung eine harte Geduldprobe. Die anderen Fälle untersuchte ich noch weiter und will später über meine Befunde noch genauer berichten.

VERTEILUNG DER SPIROCHAETE PALLIDA IM GEHIRN DER PARALYTIKER UND BEZIEHUNGEN ZU DEN EINZELNEN STRUKTURELEMENTEN.

Die Verteilung der Spirochäten im grossen und ganzen mit Bezug auf die Gehirntopographie zu erörtern, bin ich nicht in der Lage, da es nicht meine Absicht war, die Untersuchungen speziell nach dieser Seite hin zu richten. Ich kann nur im allgemeinen sagen, dass der Gyrus frontalis, der Gyrus rectus und die Regio Rolandi hauptsächlich für die gegenwärtigen Untersuchungen ausgewählt wurden, und die oben erwähnten Befunde zeigten. In einigen Fällen wurden auch der Gyrus hippocampi, das Ammonshorn und andere Regionen untersucht, in einem Falle fanden sich die Spirochäten in allen Teilen, aber in weit geringerer Zahl, als in den motorischen Zentren.

Die Pallida findet man häufiger und zahlreicher in der Hirnrinde als in der weissen Substanz. In einigen Schnitten sieht man sie in Gruppen von mehr oder weniger zahlreichen Individuen regelmässig zwischen den Nervenzellen und den Neurogliafasern verteilt. In anderen Fällen sieht man vereinzelte Exemplare über weitere Strecken hier und da verstreut. Nach Gegenfärbung der Gehirnschnitte mit Toluidinblau oder Thionin lässt sich beobachten, dass ein oder mehrere Spirochäten sich eng an eine Pyramidenzelle anlagern, und dass in einigen Fällen ein Teil des Pallidakörpers sich dem Zytoplasma der Nervenzelle zu inserieren scheint. Zuweilen liegen die Parasiten in den perineuralen Räumen längs des Verlaufes der Achsenzyylinderfortsätze. Oft auch sieht man unregelmässige amorphe Niederschläge in der Nähe solcher Zellen.



vermutlich exsudativer Natur. Die mit der Pallida infizierten Nervenzellen zeigen Degenerationerscheinungen als: Unregelmässigkeiten in der Form, Konturverzerrung, Schwellung oder völlige Auflösung des Kernes sowohl als der Zellfortsätze. Nur ganz selten sieht man die Pallida in der Nähe von Blutgefässen und fast niemals in den Gefässwandungen. Ich habe die Pia mater untersucht, konnte aber hier das Vorhandensein der Pallida nicht mit Sicherheit feststellen.

#### BEMERKUNGEN ZUR TECHNIK.

Bevor ich die Methode beschreibe, mit der ich recht gute Resultate erzielte, möchte ich noch hervorheben, dass die ältere Levaditi-methode sich oft gut bewährte. Freilich folgt die Methode, der ich mich für meine erste Serie von Untersuchungen bediente, nicht ganz streng der ursprünglichen Vorschrift, und waren die zur Imprägnierung bestimmten Stücke viel dicker geschnitten, als es von Levaditi empfohlen war. Macht man z. B. bei der Imprägnation paralytischer Hirnteile die Stücke bis zu 5, auch mehr Millimeter dick, anstatt sie nur 2 mm dick zu schneiden, so erweist sich dies bezüglich des Endergebnisses der Silberbehandlung oft sehr zweckmässig, da man im Innern so behandelter Stücke immer einen weniger tief imprägnierten Bezirk auffinden kann, innerhalb dessen die Pallida sich von den weniger tief imprägnierten Neurogliafibrillen auf das schönste abhebt. Eine andere Tatsache, der im allgemeinen wohl nicht streng genug Rechnung getragen ist, lässt sich dahin formulieren, dass in auch nur mit Formalin allein fixierten Präparaten die Neurogliafibrillen sich mit der Silberbehandlung zwar schön imprägnieren, dass die *Spirochaete pallida* aber unter solchen Verhältnissen niemals die Silberimprägnierung annimmt, gleichgültig wie lange und bei welcher Temperatur man die Stücke im Silberbad belässt. Für die Imprägnierung der Pallida ist eine vollkommene Nachfixierung der Gewebestücke in Alkohol absolut notwendig. Der allererste Schritt also, der behufs erfolgreicher Darstellung der Pallida in den Geweben des Zentralnervensystems getan werden muss, ist: völlige Fixierung der Stücke erst in Formalin und dann in Alkohol bevor man zur Imprägnierung schreitet.

Ein zweiter wesentlicher Faktor ist die Tatsache, dass langes Einwirken des Formalin die Beizfähigkeit der Neurogliafibrillen herabsetzt, während es die der Pallida erhöht. Bei Gehirnen, die wenigstens schon ein Jahr lang in Formalin aufbewahrt worden waren, gewinnt man also mehr elektive Färbung. Doch selbstverständlich geben auch frische Spezimen oft gute Resultate.

Auf Basis dieser Beobachtung formulierte ich folgende Methode, die sich mir in praxi auch ganz zweckmässig erwies.

Von einem in 10 Proz. Formalin gehärteten Gehirn wird von der Gegend des Gyrus frontalis, des Gyrus rectus oder irgend einer anderen Region ein 5—7 mm dickes Scheibchen geschnitten und zunächst in einer aus 10 Proz. Formalin, 10 Proz. Pyridin, 25 Proz. Azeton, 25 Proz. Alkohol und 30 Proz. Aqua dest. bestehenden Lösung bei Zimmertemperatur 5 Tage lang belassen. Darauf folgt gründliches Auswaschen mit destilliertem Wasser, 24 Stunden lang. Nun werden die Stücke in 96 Proz. Alkohol übertragen, in dem sie 3 Tage verbleiben (sehr wichtig) und dann wieder gründlich 24 Stunden lang ausgewaschen. Hier-nach behandelt man die Stücke in dunklen Flaschen in folgender Weise:

1. Bad in 1,5 Proz. Silbernitratlösung, entweder 3 Tage bei 37° C oder 5 Tage bei Zimmertemperatur.
2. 2 stündiges Auswaschen in destilliertem Wasser.
3. Reduktionsbad in 4 Proz. Pyrogallussäurelösung, der man 5 Proz. Formalin zugesetzt hat (24—48 Stunden bei Zimmertemperatur).
4. Gründliches Auswaschen in destilliertem Wasser.
5. Uebertragen in 80 Proz. Alkohol auf 24 Stunden.
6. 95 Proz. Alkohol, 3 Tage lang (täglich erneuern).
7. Absoluter Alkohol, 2 Tage lang.
8. Xylol, Xylol-Paraffin, Paraffin.

Es empfiehlt sich, die Schnitte aus verschiedener Tiefe der Objekte zu entnehmen, um so desto sicherer die bestimmprägnierte Zone zu treffen. Dem Grade der Imprägnierung entsprechend, die in den verschiedenen Spezimen der Gehirne ganz erheblich variieren kann, wird auch die Dicke der einzelnen Schnitte verschieden bemessen werden müssen. 3 Mikren schneide ich gewöhnlich, oft aber sind 5 vorteilhafter, man hat dann mehr Aussicht, die Pallida in einem gegebenen Bezirk zu entdecken. Sehr zweckmässig scheint es, ein beliebiges Stück syphilitischen Gewebes, das sicher zahlreiche Pallidae enthält, mitzuimprägnieren, um so für den Erfolg der Imprägnierung der Nervengewebe einen Indikator zu haben.

Fällt die Imprägnation gut aus, so erscheinen alle Gewebelemente des Gehirns schwach gelb oder gelblich braun, die Pallidakörper aber tief schwarz. Zuweilen färben sich die Neurogliafasern sehr deutlich, untersucht man diese bei künstlicher Beleuchtung, so erscheinen sie jedoch bräunlich gefärbt, niemals schwarz. Schnitte, in denen die Neurogliafasern gleichfalls schwarz erscheinen sollten, mag man ruhig verwerfen: sie eignen sich nicht zum Auffinden der Pallida und nur selten ist es mir trotzdem geglückt, auch in solchen Schnitten noch die Pallida zufriedenstellend nachzuweisen. Bei der Durchmusterung der

Schnitte beginne ich in der Regel in der blasser gefärbten zentralen Zone und verbreite mich allmählich nach den meist stärker imprägnierten Kanten zu.

All das oben Gesagte bezieht sich im gleichen auf die Rückenmarksschnitte von Fällen der Tabes. Man stellt sich ca. 2 cm lange Segmente des Rückenmarks her und fertigt Längsschnitte an.

Auf diese Art konnte ich die *Spirochaete pallida* in den Gehirnen von Fällen der progressiven Paralyse und in den Rückenmarksspezimen der Tabes dorsalis nachweisen. Prozentualiter stellten sich die positiven Befunde auf zirka 25 Proz. in den Fällen der Paralyse, während nur einer von den 12 Tabesfällen bislang einen positiven Befund aufwies. Es mag seltsam erscheinen, dass nicht alle Nervengewebe mit positivem Erfolg auf Spirochäten untersucht werden konnten, doch muss man auch in Betracht ziehen, dass die Fälle meiner Serie nicht völlig erschöpfend durchmustert wurden, da doch in der Mehrzahl derselben nur eine mehr oder weniger begrenzte Region der Hirnrinde nachgesehen wurde, und von diesen schliesslich nur eine verhältnismässig beschränkte Auswahl von Schnitten. Ich zweifle nicht daran, dass bei einer vollkommen und peinlich genau ausgeführten Untersuchung eines jeden Falles sich die Spirochäten schliesslich auch überall finden werden. Die Langwierigkeit der Technik hinderte mich, die Untersuchung noch eingehender zu gestalten, und ich hoffe, dass andere die in den eben mitgeteilten Ergebnissen noch klaffende Lücke ausfüllen werden.

Die Pathogenese der für die progressive Paralyse und die Tabes dorsalis charakteristischen Läsionen war nie ganz einwandfrei klar; nachdem jetzt aber die Syphiliserreger im Sitz der pathologischen Veränderungen selbst nachgewiesen sind, mag man wohl annehmen, dass diese Läsionen unmittelbar auf die Gegenwart der Pallida bezogen werden müssen. Im allgemeinen charakterisieren sich die Veränderungen im Gehirn als eine durch die invadierten Spirochäten hervorgerufene chronische parenchymatöse Enzephalitis. Der Grund dafür, dass die therapeutische Beeinflussung dieser Form der Spirochätose durch die heutzutage üblichen Heilmethoden so wenig befriedigende Resultate aufweist, mag wohl darin zu suchen sein, dass die Mikroorganismen, die, wie dies an den Gehirnschnitten ersichtlich ist, in der Tiefe des Organparenchyms liegen, gegen den Angriff spezifischer Medikamente wohl geschützt sind, während die

beiden gewöhnlichen Formen der Syphilis des Zentralnervensystems durch ihre Lage in der Nähe von Blutgefäßen therapeutischer Einwirkung besser zugänglich sind.

Mit dem Auffinden der *Spirochaete pallida* in den Fällen der sogen. Parasyphilis beginnt ein Dämmerlicht auf dies gigantische Problem der Therapie zu fallen<sup>3</sup>). Haben wir doch gegen die wohlbekannten Formen der manifesten Syphilis, deren Erreger dieser Organismus ist, im Salvarsan und Neosalvarsan eines der machtvollsten Kampfmittel in der Hand und ist doch der Dämon jetzt kräftiger gebannt, denn zuvor. Und kann es angesichts solcher Errungenschaften zu viel erwartet heissen, wenn wir der Hoffnung Raum geben, dass Ehrlichs Genius nochmals uns den Pfad zur therapeutischen Eroberung dieser speziellen Art syphilitischer Erkrankungen bahnen möge, die der menschlichen Gesellschaft nur allzutiefen Schaden zugefügt haben und gegen welche wir bislang völlig hilflos dastehen?

<sup>3</sup>) Flexner: Local Specific Treatment of Infections. Harvey Lectures, 1911—12, pag. 17. Lippincott Co., Philadelphia.

## THE RESULTS OF THE SERUM TREATMENT IN THIRTEEN HUNDRED CASES OF EPIDEMIC MENINGITIS.\*

By SIMON FLEXNER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

### I.

The serum treatment of epidemic meningitis, developed during the prevalence of the pandemic beginning in 1904 and not yet wholly at an end, has now been subjected to a rigorous test extending over a period of six years and embracing many different countries. The results of this trial have thus far been published in part only. The present publication deals with the analysis of the histories of cases, about 1,300 in number, that were treated with the antimeningitis serum prepared and distributed by the Rockefeller Institute. The histories have been collected not only from many different parts of the United States and Canada but also from several European and Asiatic countries to which the serum was sent. The large number of the cases and the diversity of the circumstances under which the serum came to be administered give to the following presentation a significance greater perhaps than that afforded by a strictly local report.<sup>1</sup>

### BIOLOGICAL DISTINCTIONS AMONG MENINGOCOCCI.

The bacteriological cause of epidemic meningitis is admittedly *Diplococcus intracellularis*. Formerly no fundamental biological distinction was made between meningococci; now differences of

\* Received for publication, March 1, 1913.

<sup>1</sup> The 1,300 cases include the 712 cases previously analyzed (*Jour. Am. Med. Assn.*, 1909, liii, 1443). The number of physicians and hospitals making the reports is too great to enable me to thank them individually. In a previous paper (*Jour. Exper. Med.*, 1908, x, 690) many acknowledgments were made, to which I desire now to add the name of Professor A. Netter, of Paris, for his invaluable services.

virulence for animals, degree of digestibility in leucocytes, and power of resistance to solution by immune serum are being recognized. The pathogenicity of the meningococcus for laboratory animals is not great, but by the intraperitoneal injection of young guinea pigs the virulence can be tested and different strains more or less pathogenic can be distinguished. Some strains maintain a given virulence for a year or more and others lose this quality very soon after being obtained from human sources. Rarely a strain that shows no virulence for guinea pigs takes on suddenly a heightened activity. There is no sharp agreement in pathogenicity between strains that are virulent for guinea pigs and those virulent for monkeys or man. Some strains, but not all, can be adapted by successive inoculation to guinea pigs and to monkeys.

Opsonic experiments have disclosed the fact that meningococci derived from different sources are digested by leucocytes with varying facility. The kind of leucocytes employed appears unimportant and even the sera, whether normal or immune, play no obvious distinguishing part; the variations depend upon the strain of meningococcus. All meningococci are readily phagocyted in the presence of serum and dissolved ultimately by leucocytes; but while many strains are quickly digested, a small number resist disintegration far more tenaciously. In estimating the content of opsonizing substances in different samples of antimeningococcic sera the strains digesting slowly should be chosen.

Whether in meningitis in man a similar variation among meningococci occurs naturally can only be inferred. Exudates, as obtained from different cases of epidemic meningitis, differ in number and distribution of the contained meningococci. At one time the number of meningococci is small and at others large, and sometimes the meningococci lie chiefly within cells, while at others they occur mainly free in the fluid.

Under the influence of the antimeningitis serum injected into the subdural space the diplococci come, as a rule, to lie more and more within the leucocytes. As recovery from meningitis progresses, even where no antiserum has been employed, a corresponding phenomenon is noted. Moreover, the meningococci phagocyted under the influence of the serum undergo an accelerated disintegra-

tion. In how far resistance to solution in leucocytes is due to the quality of fastness of the meningococcus to the action of the intra-leucocytic enzymes is not certainly known; nor is it known definitely that the less readily englobed meningococci consist also of strains that are more or less resistant to immune opsonins or bacteriotropins. But indications exist supporting the view that certain examples of epidemic meningitis in man which respond imperfectly to the therapeutic action of the serum are caused by strains of meningococci fast to the antiserum employed. These strains tend to remain persistently extracellular and to multiply freely in the presence of the antiserum, even under conditions of its high concentration in the subdural space. The cases of human infection with the supposedly resistant strains fail not only to respond to the therapeutic action of the antiserum but can be inferred also not to ameliorate spontaneously and thus tend to a fatal termination. Certain biological differences have been noted among meningococci from different sources. These relate to the readiness with which they can be cultivated, to survival in cultures, and fluctuation in power to ferment carbohydrates. The variations in opsonic and leucocytic reactions would seem to be of a more fundamental character, just as they are of greater significance in respect to the pathogenic power of the meningococcus and to better control of epidemic meningitis by the employment of the antimeningitis serum.

#### MORTALITY OF EPIDEMIC MENINGITIS.

The establishment of the bacterial cause and the perfection of lumbar puncture made it possible, as never before in a widely spread epidemic of meningitis, to ascertain accurately the degree of its fatality. Mortality estimates based on earlier epidemics are far less trustworthy since they obviously could not have rested on such certain evidence of specific disease. The pandemic of 1904-9 afforded, therefore, the first opportunity to determine the actual mortality of epidemic meningitis as it affects countries and peoples of various nationalities and widely different social customs. Accurate knowledge of the mortality becomes imperative once we undertake to determine the value of the serum treatment. Com-

prehensive and accurate figures are available from the United States and Canada, Great Britain and Ireland, Germany, France, Belgium, and Italy, and Asia and Africa. The facts to be given immediately relate exclusively to the disease as it prevailed either before the serum treatment was introduced, or where it had not yet been applied.

In the period from 1904 to 1909 epidemic meningitis prevailed over a wide territory in the United States and Canada, and after an intermission reappeared in the winters of 1911-12 and 1912-13 in the Southwestern States. The fatalities recorded at several places are as follows: Greater New York City, 73 per cent.; Boston, 69 per cent.; Hartford, 76 per cent.; several cities in Ohio, 70 to 90 per cent.; Porterville, California, 90 per cent. During 1911-12 more than 1,500 cases arose in Texas and Louisiana among which (exclusive of cases treated with serum) the mortality was 75 per cent. or more.

The epidemic in Great Britain was equally severe. The mortality reports include Leith, 74.7 per cent.; Edinburgh, 80 per cent.; Glasgow, 74.8 per cent., and Belfast, 70 per cent.

The largest German figures relate to the Silesian epidemic. The lowest mortality reported is based on a group of 47 cases at Brieg and is 42.5 per cent., while in Breslau it was 62.3 per cent., and among the 3,085 cases collected by Flatten, 67 per cent.

The mortality in France was placed by Netter at 75 per cent.; in Belgium it is given at 77.7 per cent.; while the degree of fatality in Italy may be deduced from the figures for Milan which are 55.6 per cent.

The reports from Asia and Africa are few but they indicate severe infection. In Palestine the mortality was above 80 per cent.; in Greece (Athens) 58.6 per cent.; and in the Transvaal 74 per cent. Although accurate figures are not available, the epidemic prevailed in Java and in German East Africa.

The statement of von Ziemssen may be taken as a criterion of the views held in prebacteriological days of the severity of epidemics of meningitis. He states that the mortality fluctuates between 30 and 70 per cent., the average being about 40 per cent. There is, of course, something highly artificial in arriving at an average in



this manner, and the artificiality is brought out strikingly by the reliable figures of the recent pandemic.

There appears to have been little variation in the mortality according to the period at which the epidemic flourished, and as it affected the inhabitants of the mining districts of Northern Germany or the Transvaal, the industrial populations of Ireland or Scotland, the general population of France, the military garrisons of Belgium, the urban population of New York or Boston, the suburban population of remote districts in Ohio, California, Texas, and Louisiana, or the agricultural and artisan peoples of Greece and Palestine. Everywhere the upper limits of von Ziemssen's figures were reached or surpassed. Hence it appears that the virulence of the meningococcus was the chief factor in causing death, and differences among peoples, as represented by race and occupation, were far less conspicuous factors.

Meningococcus meningitis prevails also as a sporadic affection. The mortality of the sporadic disease is far more difficult to determine than that of the epidemic disease for the reason that vital statistics covering wide areas are difficult to obtain. Such data as are available tend to show that the sporadic disease, when bacteriologically diagnosed, is less fatal than the epidemic disease. Posterior basic meningitis, which is probably merely a variety of sporadic meningitis, has a less general distribution. This disease had claimed attention chiefly in England. The fatality of these affections may fall as low as 40 to 50 per cent.

#### MODE OF TERMINATION.

Cases of meningitis are very varied both in respect to their intensity and evolution. In rare individual instances the disease sets in severely and yet terminates in recovery, sometimes in a few days, or the onset is mild although the infection later develops an alarming gravity; while again the disease progresses slowly or continuously through weary weeks or months to end usually, but not always, in death. The cases of ordinary severity pursue a middle course, while the fulminant ones arise and terminate within a few to forty-eight hours. There remains still another variety of the disease hitherto little remarked and the frequency of which is not

known; namely, the ambulant. The subjects are commonly little ill at any time, so that recovery is the rule. Nevertheless, in rare instances death may suddenly supervene, and somewhat more often the slight illness increases in severity until the ordinary type is evolved.

Neither the symptoms nor the mode of their evolution support a prediction as to the probable outcome of the disease. Several different types have been constructed out of the variety, complexity, and degree of severity of the symptoms, but a sharp discrimination between them is often wanting in practice. For this reason observation of individual cases affords little final information concerning the nature of epidemic meningitis, and the outcome of single cases is an uncertain guide to the value of a given therapeutic measure.

Death may be considered as taking place under three sets of circumstances: (*a*) rapidly, after sudden onset of illness, as in meningitis siderans, or so called fulminating meningitis; (*b*) through a gradual intensification of the symptoms and after a duration of from six to ten days; (*c*) slowly, in the course of which the acute stage has been survived and after the condition has become chronic.

Recovery, also, when it occurs is not by a single mode of termination, but by at least two modes. The transition to convalescence and recovery is generally by a process of gradual amelioration of the symptoms, or lysis. Sometimes, but more rarely, the transition takes place abruptly by a sudden or critical cessation of the symptoms that follow quickly upon the onset of the infection or after several days have elapsed.

Knowledge is lacking of the relative frequency with which the several types of meningitis occur in different epidemics. What would be particularly informing is accurate knowledge of the frequency of the extreme cases—the fulminant and the abortive, latent, rudimentary, or ambulant ones, so called. The throwing together of the abortive and ambulant cases is not strictly justified by their usually mild character, since the one sets in sharply and the other begins insidiously. While the former tends to terminate in recovery by crisis, the latter attracts attention by suddenly pass-

ing into the severe or even fulminant disease. That such mild cases of epidemic meningitis occur has now been conclusively shown by bacteriological examination of fluids removed by lumbar puncture. Exceptionally an epidemic has been composed mainly of them, as in Kehl. In many epidemics they have wholly escaped detection. Apparently their occurrence is infrequent, and excellent observers of large numbers of cases of epidemic meningitis have not encountered them.

The proportion of fulminant to ordinary cases can be inferred rather than asserted. At the beginning of a severe epidemic they appear to be more frequent than at the end. Certain epidemics have been noted for their absence. On the other hand, fulminant cases have appeared in associated groups, one case following another in rapid succession in barrack, school, or family. Some idea of frequency can be inferred from the duration of the illness preceding death. The chief danger in this inference arises from the uncertainty surrounding the period of onset of some of the cases. Among 1,481 fatal cases analyzed by Flatten, 29 died within six, 30 in from six to twelve, and 53 in from twelve to twenty-four hours of the appearance of symptoms. That is, 112, or 9 per cent., succumbed within the first twenty-four hours, while another 111 cases terminated fatally in the second twenty-four hours, giving 18 per cent. of the whole as having suffered from the fulminant disease.

#### DURATION OF THE DISEASE.

The duration may be considered according to the period elapsing before either death or recovery occurs. Among the 1,481 fatal cases just mentioned, 361, or 25 per cent., terminated in the first three days; 351, or 24 per cent., from the fourth to the seventh day; and almost 50 per cent. later than the seventh day of illness. Hence, it cannot be maintained that the survival of the first week of the infection affords great insurance of ultimate recovery. One half of the cases surviving the first week terminated between the fifth and eighth weeks, while 7 per cent. (107 cases) survived eight weeks or longer. Among Schneider's 80 fatal cases, 26, or 32.5 per cent., succumbed in the first three days; 18, or 22.5 per cent., in the next four days; and 36, or 45 per cent., later than the first week.

Viewed from the side of recovery the period of duration of the disease has fluctuated with the type. The abortive cases are said to terminate in recovery in from one and one half to five days (von Ziemssen, Strümpell). Strümpell states that the critical period falls between the fifth and tenth days, when either death results or convalescence begins. Schneider's analysis of 52 cases shows 3 recoveries from the fifth to the ninth day, 26 from the eleventh to the fortieth day, and 45 from the forty-first to the one hundred and tenth day. Rieger discharged 25 patients from the hospital on the average of the fortieth day. Heubner states that the average duration of the disease terminating in lysis is three to four to six weeks; von Ziemssen gives for favorable cases two to three weeks, and for less favorable ones three to four weeks. Holt states that in the New York epidemic of 1904-5, among 350 cases which recovered, the duration in 3 per cent. was one week or less, and in 50 per cent. five weeks or longer.

Thus we may conclude that in the recent past the tendency of epidemic meningitis has been to terminate in death, and of the fatalities about one half arose in the first week of the illness. On the other hand, among the recoveries a small percentage took place in the first week, and almost all after several weeks or months of illness.

#### INCIDENCE AND MORTALITY ACCORDING TO AGE.

Young persons are more subject than older ones to epidemic meningitis. This conclusion, arrived at by Hirsch and others, is supported by recent more accurate studies. Certain epidemics affect children almost entirely, and nurslings rather than older children (Hirsch, Heubner). Among the 2,916 cases in Silesia in 1904-5 analyzed by Flatten, 247 arose in the first, 239 in the second, 293 in the third, 334 in the fourth, and 271 in the fifth year. Hence about one half (1,384) of the cases occurred in the first five year period, one third (844) in the second, one eighth (338) in the third corresponding period, and the remainder (350) later than the fifteenth year of life. Göppert noted among 629 cases 102, or 17 per cent., in nurslings. Since in children below three years of age the symptoms of meningitis are often masked, it follows that the

bacteriological diagnosis accounts for the greater number of infants shown to be attacked during the pandemic.

The mortality is affected by the age of the attacked. Hirsch states that the disease is most fatal in children and in adults after the fortieth year. During the epidemic in New York the mortality among infants of two years and under approximated 100 per cent. Flatten's figures based on the Silesian epidemic show approximately the same result. The number of cases analyzed was 2,976. They may be divided as follows: under one year, 255 with 89 per cent. of deaths; under two years, 243 with 83 per cent. of deaths; between two and five years, 866 with 72 per cent. of deaths; between five and ten years, 920 with 62 per cent. of deaths; between ten and fifteen years, 346 with 56 per cent. of deaths; between fifteen and twenty years, 187 with 63 per cent. of deaths; between twenty and thirty years, 76 with 59 per cent. of deaths; and between thirty and seventy years, 83 with 83 per cent. of deaths.

Hence, in the past, epidemic meningitis has been most fatal to infants and adults after the third or fourth decennium, while the intermediate age periods have been somewhat more favorable to spontaneous recovery. The favorable period begins to be apparent about the fifth year and endures until the twentieth or thirtieth year, when it diminishes with increasing rapidity as age advances.

#### COMPLICATIONS AND SEQUELS.

We shall consider briefly the chief complications and sequels only. The common secondary infections of the respiratory tract, etc., will be neglected. In some instances the meningococcus causes a complicating pneumonia, endocarditis, or serositis exclusive of the joints; in very rare instances it has produced general infection or septicemia.

Of greater importance are the involvements of the central nervous system, peripheral nerves, and sense organs in the infective process. True motor paralyzes occur infrequently and permanent ones are rare. Of the nerves of the head the facial and hypoglossal are oftenest affected. The frequency has varied with different epidemics and the complication has been noted in abortive as well as severe cases and tends to arise at the end of the first week. Hemi-

paresis, a rare condition, affecting an arm and less often a leg, comes on usually later than the second week. These paralyzes, regarding which figures are scant, are attributed to focal lesions in the brain and spinal cord extending inward from the meninges.

The ear complications are numerous. Both the middle and internal ear may be affected. Inflammation of the middle ear is frequent and increases with the duration of the illness. Thus 35 per cent. of the inflammations arise in the first, 70 per cent. in the second, and 59 per cent. in the fourth to the sixth week (Göppert). Recovery tends to be complete. Inflammation of the internal ear is far more serious. The frequency varies in different epidemics. Percentages range from 33 (Mannkopff and Heller), 16 (Göppert), and 12 (Bauer). Probably 12 per cent. approximates the average among large numbers of cases (Göppert). The involvement usually occurs early in the attack and arises even in the abortive disease. Both ears tend to be affected and hearing is often already lost when the patients enter the hospital. Among sixty-four cases Moos observed affections of one side in three. When the accident occurs in infancy deaf-mutism results. Following epidemics of meningitis the number of deaf-mutes in institutions has greatly increased.

The eyes may suffer slightly or severely, and injury may be temporary or permanent. Muscular affections are common (12 per cent.), ptosis is rare, and pupillary irregularities are frequent (24 per cent.); all tend to be temporary, except ptosis. Iritis and irido-choroiditis, retinitis, and periretinal hemorrhages all arise but often disappear without causing permanent injury. Keratitis, with or without ulceration of the cornea, occurs, followed either by recovery or loss of the eye. A condition of basilar amaurosis, from which slow recovery has taken place, has also been described (Göppert). The most serious complication is metastatic ophthalmia, due to the meningococcus, affecting one eye or both, and leading to destruction of the ball. Eye complications have been a variable factor in different epidemics. Göppert reports 14 instances of metastatic ophthalmia (affecting both eyes in 4, and one eye in 10 patients) among 136 cases (10 per cent.). Uthoff states that among 110 Silesian cases lesions of the optic nerve occurred in 17, metastatic

ophthalmia in 4, and keratitis in 3. Morax noted iridocyclitis in from 3 to 6 per cent. of cases during the Parisian epidemic.

The higher intellectual centers of memory, intelligence, and speech suffer also, but while slight degrees of affection from which recovery takes place are common, only rarely does permanent incapacitation result. Actual mental defects of permanent character arise, according to Göppert, in from 1 to 3 per cent. of the recovered. Looft reports that among 539 Norwegian idiots, 3.7 per cent. were caused by epidemic meningitis.

Finally, hydrocephalus arises very often, especially among the young. In Silesia its occurrence was noted in one eighth of all cases. Recovery may occur without mental defect even where the degree is considerable. Deafness and hydrocephalus are frequently associated, but not as cause and effect.

## II.

We may now proceed to consider what changes have been effected in the course and consequences of epidemic meningitis by the employment of the antimeningitis serum. The serum began to be employed in 1906-7, first locally and later more generally. Thus it failed to be used in certain places because the epidemic wave had passed, and in other places its employment was begun at the middle period or height of the epidemic, according to circumstances. There occurred coincidentally, almost without exception, cases of equal severity in which the serum was not administered. These served as checks, or controls. The mortality in them did not vary essentially from the figures already given for different countries (page 251). The analysis to follow is based upon cases treated in America, Europe, Asia, and Australia with the serum which was prepared at the Rockefeller Institute by a uniform method and widely distributed, and they therefore present at least this common factor. The chief variable was the experience or lack of experience in those charged with the administration of the serum, which is sometimes reflected in the results achieved. But since this factor will always operate in some degree, the results probably approach the normal.

The high degree of variability of the clinical course of epidemic

meningitis has been pointed out; hence it will not be as profitable to study the influence on individual cases as to consider the effects on the mortality and some other objective features of the disease.

#### MORTALITY OF SERUM-TREATED CASES.

The number of cases to be subjected to analysis is 1,294. They have not been selected and a single exclusion has been practised. In order to deny no one the benefits of the treatment where available, a small number of hopeless and dying persons were injected. When they survived the injection merely a few hours—less than twenty-four at the maximum—they were not included. The fulminant cases have been included in the analysis and, as will appear, they now seem not to be without the sphere of influence of the serum. The general mortality is shown in table I.

TABLE I.  
*Mortality of Serum-Treated Cases.*

No. of cases.	Recovered.	Died.	Per cent. died.
1,294	894	400	30.9

That the period of injection in the course of the disease affects the fatality has been previously shown.<sup>2</sup> The larger number of cases has been analyzed from this point of view and in accordance with the arbitrary periods previously employed. The results are shown in table II.

TABLE II.  
*Mortality according to the Period of Injection of the Serum.*

Period of injection.	No. of cases.	Recovered.	Died.	Per cent. recovered.	Per cent. died.
1st to 3d day . . . . .	199	163	36	81.9	18.1
4th to 7th day . . . . .	346	252	94	72.8	27.2
Later than 7th day . . . . .	666	423	243	63.5	36.5
Totals . . . . .	1,211	838	373	69.2	30.8

The pronounced influence of the serum is strikingly shown by these tabulations which serve to confirm the earlier figures given.<sup>3</sup>

<sup>2</sup> Flexner, S., *Jour. Exper. Med.*, 1908, x, 699.

<sup>3</sup> Flexner, S., *ibid.*, pp. 700-1.



In some instances the precise period of first injection of the serum was not stated in the histories, which explains the smaller number of cases analyzed in table II. This table not only establishes the fact that the earlier the serum injections are begun the better the results, but it emphasizes the too common delay in making the diagnosis of epidemic meningitis or of bringing the majority of cases under the specific treatment. Less than one sixth of the cases had the benefit of early injection, and one half received the first injection later than the first week of illness. Doubtless as the serum is made more generally available and as confidence in its value extends, these disadvantages will be more and more removed.

That the mortality is affected by the age of the patient is commonly admitted. The influence of the serum on patients of different ages is shown in table III.

TABLE III.

*Mortality according to Age.*

Age.	No. of cases.	Recovered.	Died.	Per cent. recovered.	Per cent. died.
Under 1 yr. ....	129	65	64	50.4	49.6
1 to 2 yrs. ....	87	60	27	69.0	31.0
2 to 5 yrs. ....	194	139	55	71.6	28.4
5 to 10 yrs. ....	218	185	33	84.9	15.1
10 to 20 yrs. ....	360	254	106	70.6	29.4
Over 20 yrs. ....	288	180	108	62.5	37.5
Age not given. . .	18	11	7	61.1	38.9
Totals. ....	1,204	804	400	66.1	33.9

TABLE IV.

*Mortality according to Age and Period of Injection.*

Age.	Injected 1st to 3d day.				Injected 4th to 7th day.				Injected later than 7th day.			
	No. of cases.	Re-cov-ered.	Died.	Per cent. died.	No. of cases.	Re-cov-ered.	Died.	Per cent. died.	No. of cases.	Re-cov-ered.	Died.	Per cent. died.
Under 2 yrs. ....	13	12	1	7.7	37	28	9	24.3	159	81	78	49.1
2 to 5 yrs. ....	30	24	6	20.0	66	49	17	25.8	93	63	30	37.3
5 to 10 yrs. ....	55	49	6	10.9	69	61	8	11.6	77	61	16	20.8
10 to 20 yrs. ....	67	58	9	13.4	106	73	33	31.1	171	115	56	32.7
Over 20 yrs. ....	34	20	14	41.2	65	39	26	40.0	164	101	63	38.4
Totals. ....	199	163	36	18.1	343	250	93	27.1	664	421	243	36.6

Table IV gives an analysis of the data of the preceding tabulation according to the period of the disease at which the serum injections were begun.

The age factor affects the outcome of the serum treatment just as it affects spontaneous recovery, only its influence is even more marked in the former. The most favorable cases for the specific treatment fall between the five and ten year period and the least favorable ones fall in the period above twenty years. The favorable period doubtless extends to and even beyond the twentieth year although the analysis given fails to make an exact discrimination for these ages. To subdivide farther the periods risks increasing the statistical error inherent in small numbers; but the reports of certain hospitals covering cases arising during the third decennium indicate no great decrease in response to the serum. What is particularly impressive is the change wrought in the fatality among infants, and while the astonishing result for the first injection period can scarcely be maintained through the vicissitudes of large numbers of cases, yet the outlook is highly promising. The exact coincidence of the favorable ages for spontaneous recovery and for recovery under the serum treatment indicates that the serum provides artificially the means that the body itself employs when it can fabricate them to suppress the infection, and that two factors, one provided by the body and the other by the serum, coöperate in the end result. The change wrought in the manner of termination of the disease enforces the same conclusion.

#### MANNER OF TERMINATION AND DURATION.

The usual mode of spontaneous termination of epidemic meningitis is by gradual subsidence of the symptoms, or lysis, and the exceptional mode is by abrupt cessation, or crisis. While precise figures showing the number of cases subsiding by crisis in ordinary epidemics are not available, yet it can be said that it has never been observed to be large and that sometimes this class of cases has been wholly absent. The histories of 830 cases embraced in our tabulation permitted of a judgment as to the manner of termination, and it was ascertained that in 30 per cent. the cessation was abrupt, or by

crisis. In our previous tabulation of 400 cases<sup>4</sup> the percentage was placed at 25, which apparently was too low.

A consideration of the manner of recovery according to the age groups brings out some facts of suggestive interest. The data are given in table V, from which it will be seen that among cases of all ages termination by crisis and by lysis occurs about equally when the injection of the serum was begun in the first three days of illness, while when the injection was begun later the number of cases terminating by lysis rose steadily. One other point is indicated by the tabulation; namely, that the proportion of cases that resolve by crisis is highest in the age periods more favorable, and lowest in those less favorable to recovery, whether spontaneously or through specific treatment.

TABLE V.

*Recovery by Lysis or Crisis according to Age Periods.*

	1st to 3d day.	4th to 7th day.	Later than 7th day.	Total.
Under 1 yr.				
By lysis.....	3	6	36	45
By crisis.....	2	6	8	16
1 to 2 yrs.				
By lysis.....	4	10	26	40
By crisis.....	3	6	8	17
2 to 5 yrs.				
By lysis.....	10	31	53	94
By crisis.....	14	18	10	42
5 to 10 yrs.				
By lysis.....	26	43	43	112
By crisis.....	23	18	19	60
10 to 20 yrs.				
By lysis.....	28	46	92	166
By crisis.....	30	27	23	80
Over 20 yrs.				
By lysis.....	11	22	85	118
By crisis.....	9	17	14	40

With the manner of termination is obviously bound up the period of duration of the active symptoms of the disease. Epidemic meningitis pursuing its usual or typical course passes through a series of reinfections of the cerebrospinal meninges, whence its intermittent or remittent character. To determine, therefore, just

<sup>4</sup> Flexner, S., and Jobling, J. W., *Jour. Exper. Med.*, *loc. cit.*, p. 690.

what constitutes a relapse of the infection is sometimes difficult or even impossible. The most trustworthy guide, next to long and complete intermission of symptoms, is afforded by bacteriological examination of the cerebrospinal liquid, since the meningococci tend not to disappear wholly during the intermissions, while they do so disappear between true relapses. Having in mind these criteria we have separated from the 1,294 cases 56 that showed relapses after greater or less intervals of time. Prompt resumption of the serum injections often but not invariably sufficed to control and suppress the reinfections. Of the 56 cases 40 recovered and 16 died. Among them the mortality was 28.6 per cent., indicating that the relapses respond somewhat less well to the serum than the primary infections.

#### RESPONSE OF RESISTANT OR FAST STRAINS.

In some instances the meningococci having been at first readily controlled by the injections appear to develop resistance or fastness to the serum. This has been noted in certain cases of relapse going on to a fatal termination. Hence under special circumstances the meningococci seem to acquire a serum-fastness that thwarts its specific action. A variety of serum-fast meningococci exists in a small number, at least, of cases of epidemic meningitis before the serum has been applied. This original fast strain seems not to be of common occurrence and its biological properties have not been minutely studied. It is not known whether serum fastness and indisposition to opsonization and leucocytic digestion go hand in hand or not. Nor is it known whether merely one or many fast strains distinct from one another exist in nature. Information on these points is highly desirable, as is the utilization of fast strains of the meningococcus in the preparation of the antimeningitis serum. Should a true polyvalent serum of high titre carrying immune bodies for the fast strains be developed it is probable that the mortality percentages would come to be depressed still further.

#### COMPLICATIONS AND SEQUELS.

The number of cases here analyzed should serve to indicate the frequency with which the usual complications and sequels may be met with under the serum mode of treatment. Our earlier tabula-

tion suggested that the recoveries would, as a rule, be complete and the one severe sequel that would still remain, although in diminished degree, is deafness. It is impossible to know whether the histories returned noted all the sequels, but it is probable that the severer ones, which we are considering, were uniformly recorded. I have tabulated the reported instances of impaired hearing and vision and arthritis in relation to the day of the disease on which the first injection of serum was made. The circumstance that sometimes the defect already existed when the injection was made can be disregarded, since, as this fact cannot always be established and as a specific form of treatment may be presumed to be capable, at times, of suppressing the secondary localizations of the meningococci, no exclusion should be practiced.

TABLE VI.

*Impaired Hearing and Vision and Arthritis in Serum-Treated Cases.*

Day of disease of first serum injection.	1st day.	2d day.	3d day.	4th day.	5th day.	6th day.	7th day.	2d week.	3d-8th week.	Totals
No. of cases in which deafness occurred . . . . .	1	15	6	5	3	3	1	7	4	45
No. of cases in which impaired vision occurred . .	1			2	2			3	4	12
No. of cases in which arthritis occurred . . . . .		5	3	1				2		11

The instances of arthritis all recovered, as is the rule; and the recovery could be hastened by injecting the serum directly into the inflamed joint. Deafness was complete in 39 and partial in 6 of the 45 instances. Blindness occurred 3 times, some degree of impairment of vision 6 times, iridocyclitis, choroiditis, and metastatic ophthalmia (affecting one eye) once each. Impaired mentality occurred 3 times; in one instance the state is described as imbecile. Muscular paralyses were recorded in 11 histories as follows: strabismus in 5, paralysis of the lower extremity in 3, of the face in 2, and of the shoulder in 1. Deafness and impaired vision were combined in 2 cases, imbecility and impaired vision and arthritis and choroiditis occurred in 1 case each.

The severe and permanent sequels are those resulting from affections of the internal ear and the essential structures of vision. The former arose in about 3.5 per cent. of the serum-treated cases.

This constitutes a marked reduction of the percentages that have been observed among the spontaneously recovered; whether they can be still further diminished by a more prompt and vigorous employment of the serum cannot be predicted.

#### DISCUSSION.

The foregoing analyses demand merely a brief discussion since they carry their own forceful argument. They show indubitably that the mortality of epidemic meningitis can be greatly reduced by the application of the specific serum treatment and the extent of the reduction is determined by two main factors: the period of the disease at which the subdural injections of the serum are begun and the ages of the persons affected. In view of the fact that the average mortality during the pandemic was approximately 70 per cent., the gross reduction was somewhat less than two thirds. On the other hand, the statistics presented indicate that general early diagnosis and prompt institution of the serum treatment are capable of still further depressing the mortality.

Although at first regarded as doubtful it now appears that fulminant cases of epidemic meningitis are not wholly without the sphere of beneficial influence of the serum. This conclusion rests, first, upon specific statements of recovery of such cases under the influence of the serum, and, next, upon the definite change wrought by it in the mortality of the disease during the first three days of prevalence which is the period during which the fulminant cases terminate in death. Flatten's figures (page 254) and the figures given in table II afford a basis for comparison covering this point.

Undoubtedly it is the ordinary type of epidemic meningitis that responds best to the specific treatment. This fact is shown not only by the general reduction in mortality, but also by the common alteration of the manner of its termination from lysis to crisis. It is just this transformation and the general shortening of the whole course of the disease and the consequent suppression of the chronic form of the infection that bring conviction of the value of the serum not only by reason of statistical presentations but even more cogently to the hospital physicians who have large experience with the usual course and tendencies of the malady.

Other objective criteria of the action of the serum exist. One of the most conclusive is the effect exerted upon the meningococci through which multiplication is arrested and phagocytosis and intra-leucocytic digestion accelerated. This impressive evidence of the action of the serum has been recorded many times by different physicians in the histories upon which this paper is based

The complications and sequels are reduced in number. Indeed, the number of permanently injured among the serum-treated has become very small. Of all the severe sequels deafness has remained least influenced. Unhappily, injury to the internal ear takes place very early and sometimes before the diagnosis of meningitis has been made. In view of the great reduction in other severe effects we are permitted to hope that even this one may be diminished by a more uniformly early application of the serum. The arthropathies have not only been reduced in frequency but they have been shown to be amenable to direct injections of the serum. Undoubtedly the tendency to hydrocephalus in the young has been diminished, and the intraventricular injection of the serum has operated in several instances to abolish infection and inflammation of the cerebral ventricles and to reestablish communication between the ventricles and the subdural space of the spinal cord. Recovery, therefore, has been rendered complete.

### III.

#### ADDENDUM.

In the preceding presentment the value of the serum treatment is based wholly upon the experience gained by the Rockefeller Institute during the several years it was engaged in preparing and distributing the antimeningitis serum. Three reports on the subject have been issued at different times; this report is the final one, since the Institute no longer prepares the serum and is collecting no more histories of cases treated with it. During the period of several years in which the serum was on trial, reports from other countries have also been published. The most important possibly are those of Levy and Netter. They support the value of the serum treatment, as do the many briefer ones published. Within the past

two years two widely remote epidemics of meningitis came under the serum treatment, of which the results are now available for study. One occurred in Greece, the other in the Southwest of the United States. Of the former a set of figures from Athens has been published. The serum employed was chiefly that prepared at the Rockefeller Institute and the cases were treated at the Annunciation Hospital by Dr. Chrestomanós. The tabulation which follows gives the main important facts which are in complete accord with the corresponding data of our previous tabulation (table II).

TABLE VII.

*Serum-Treated Cases of the Grecian Epidemic, 1911-12.*

Period of injection.	No. of cases.	Recovered.	Died.	Per cent. died.
1st to 3d day . . . . .	100	87	13	13.0
4th to 7th day . . . . .	54	40	14	25.9
Later than 7th day . . . . .	32	17	15	47.0
Totals . . . . .	186	144	42	22.6

During the past two winters (1911-12, 1912-13) Texas, Louisiana, and still other southern and western states have suffered in some degree from an extension or reappearance of epidemic meningitis. The serum was employed in many of the cases, and the reports from Texas<sup>5</sup> and Louisiana are available for 1911-12. They are specially valuable since they indicate what can be accomplished by the employment of the serum not only inside hospitals but outside and, as often happens, under the disadvantages of poor surroundings and, doubtless, by physicians without previous experience in the performance of lumbar puncture.

The total cases reported from the cities (exclusive of Dallas) and counties in Texas numbered 1,956. Of them 562 received no serum and the mortality was 77 per cent. 1,394 were given one or more injections of the serum and the mortality was 37 per cent. Since the conditions for the care of patients tend to be better in the cities than in country districts, any treatment is likely to be carried out more effectively in them. The figures for three cities that suffered heavily in the epidemic follow.

<sup>5</sup> Sophian, A., *Experimental Cerebrospinal Meningitis*, St. Louis, 1913.



Galveston: 83 cases treated with the serum; mortality 24 per cent.

Houston: 169 cases treated with the serum: mortality 27 per cent.

Dallas<sup>a</sup>: 444 cases treated with the serum; mortality 27 per cent.

Dallas<sup>b</sup>: 180 cases treated with the serum; mortality 25 per cent.

The preceding figures are uncorrected and include all cases receiving the serum without reference to the quantity given or the period of the disease when first treated. Not a few cases came under treatment when already moribund. Among the group of cases observed by Dr. Sophian 19 were in a dying condition when first given the serum and survived the injection less than twenty-four hours. Excluding these almost hopeless examples lowers the mortality among the 161 remaining cases to 15.5 per cent.

TABLE VIII.

*Shreveport Epidemic, 1912.*

	Serum-treated.				Non-serum-treated.			
	Total No. of cases.	Recovered.	Died.	Per cent. died.	Total No. of cases.	Recovered.	Died.	Per cent. died.
	176	123	53	30.1	74	11	63	85.0

*Mortality according to Race.*

	Serum-treated.				Non-serum-treated.			
	Total.	Recovered.	Died.	Per cent. died.	Total.	Recovered.	Died.	Per cent. died.
White.....	67	47	20	29.8	27	7	20	74.0
Colored.....	109	76	33	30.3	47	4	43	91.5

The data given in the official report of the Louisiana State Board of Health relating to the Shreveport epidemic may be studied from various points of view. The total cases numbered 250. The results of my analysis of the cases are presented in the tabulation. The chief facts may be stated as follows: the mortality among the non-serum-treated cases was 85 per cent., and among the serum-treated 30 per cent.; white and colored patients responded equally to the serum, but without serum the colored patients succumbed in

<sup>a</sup> Reported by Dr. Nash.

<sup>b</sup> Reported by Dr. Sophian.

greater proportion than the white. Doubtless this difference arose from the generally poorer surroundings and care of the colored race rather than from any inherent lack of resistance to the infection. As a rule, the number of injections and the amount of serum administered have been given in the report. Where the administration was obviously futile the case has been reclassified.

#### CONCLUSION.

The data brought together in this report have been gathered from a wide territory and for a period extending over several years. The antimeningitis serum was first employed in 1906 and the latest figures relating to its use included in this report were furnished in 1912. There is no longer doubt that the serum has come to be applied under conditions fairly representing all known manifestations of epidemic meningitis. Hence the test of the serum treatment may be regarded as having been a rigorous one. The initial difficulties surrounding the administration by direct subdural injection have been largely overcome and doubtless will be still further mastered. Already the serum is being successfully applied in private as well as in hospital practice. This gain will probably be reflected in a still further diminution of the mortality since early injection plays such a large part in determining the results achieved.

The 1,300 cases studied in this report are a part only of a far greater number of cases actually treated with the serum supplied by the Rockefeller Institute. It was not found possible to secure histories of all the cases treated; but there is no reason to suppose that the results of the analysis would have been essentially different if reports of a still larger number of cases had been returned. The decision arrived at is not based upon statistical computations alone, but upon objective data as well that are not readily misinterpreted.

The conclusion that follows was first stated in 1909 as the result of an analysis of 400 serum-treated cases. It is supported by the study of the larger series of cases just presented.

"In view of the various considerations presented, the conclusion may be drawn that the antimeningitis serum, when used by the subdural method of injection, in suitable doses and at proper intervals, is capable of reducing the period of illness; of preventing, in large

measure, the chronic lesions and types of the infection; of bringing about complete restoration of health, in all but a very small number of the recovered, thus lessening the serious, deforming, and permanent consequences of meningitis; and of greatly diminishing the fatalities due to the disease."

## BIBLIOGRAPHY.

- Billings, J. S., Jr., Cerebrospinal Meningitis in New York City during 1904 and 1905, *Jour. Am. Med. Assn.*, 1906, xlv, 1670.
- Bouché, G., Un nouveau cas de méningite cérébrospinale à méningocoque de Weichselbaum; sérothérapie; guérison, *Jour. de méd. de Bruxelles*, 1912, xvii, 61.
- Currie, J. R., and MacGregor, A. S. M., The Serum Treatment of Cerebro-Spinal Fever in the City of Glasgow Hospital, Belvidere, between May, 1906, and May, 1908, *Lancet*, 1908, ii, 1073.
- Discussion on Cerebro-Spinal Meningitis in the Seventy-Sixth Annual Meeting of the British Medical Association, *Lancet*, 1908, ii, 1339.
- Dunn, C. H., Animal Experimentation in Relation to Epidemic Cerebrospinal Meningitis, *American Medical Association Defense of Research Pamphlets*, 1911, No. 21.
- Feltsos, T. J., Cerebrospinal Meningitis in Greece, *Public Health Reports*, 1911, xxvi, 1006.
- Fischer, L., Klinische Beobachtungen über Meningitis cerebrospinalis und die Resultate der Behandlung mit Flexner-Serum in New York, *Arch. f. Kinderheilk.*, 1910, lii, 289; Cerebrospinal Meningitis in an Infant, Two Months Old, *New York Med. Jour.*, 1910, xci, 625.
- Flatten, H., Die übertragbare Genickstarre im Regierungsbezirk Oppeln im Jahre 1905 und ihre Bekämpfung, *Klin. Jahrb.*, 1906, xv, 211.
- Flexner, S., Experimental Cerebrospinal Meningitis and its Serum Treatment, *Jour. Am. Med. Assn.*, 1906, xlvii, 560; Experimentelle Cerebrospinalmeningitis und ihre Serumbehandlung, *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1907, xliii, 99; Contributions to the Biology of *Diplococcus intracellularis*, *Jour. Exper. Med.*, 1907, ix, 105; Concerning a Serum-Therapy for Experimental Infection with *Diplococcus intracellularis*, *ibid.*, p. 168; The Present Status of the Serum Therapy of Epidemic Cerebrospinal Meningitis, *Jour. Am. Med. Assn.*, 1909, liii, 1443; Meningokokken-Serum, in Wolff-Eisner, A., Handbuch der Serumtherapie und experimentellen Therapie, München, 1910, 142; The Local Specific Treatment of Infections, with Especial Reference to Epidemic Meningitis, *Edinburgh Med. Jour.*, 1912, viii, 389.
- Flexner, S., and Jobling, J. W., The Serum Treatment of Cerebrospinal Meningitis, *Jour. Exper. Med.*, 1908, x, 141; An Analysis of Four Hundred Cases of Epidemic Meningitis Treated with the Antimeningitis Serum, *ibid.*, 690; Kurzer Bericht über 400 Fälle von Meningitis, behandelt mit intraspinaler Einspritzung von einem Antiserum, *Zentralbl. f. inn. Med.*, 1908, xxix, 893.

- Göppert, F., Zur Kenntnis der Meningitis cerebrospinalis epidemica mit besonderer Berücksichtigung des Kindesalters, *Klin. Jahrb.*, 1906, xv, 523; Über Genickstarre, *Ergebn. d. inn. Med. u. Heilk.*, 1909, iv, 165.
- Heubner, O., Cerebrospinalmeningitis, in Eulenburg, A., Real-Encyclopädie der gesamten Heilkunde, Wien und Leipzig, 1894, iv, 425; Beobachtungen und Versuche über den Meningokokkus intracellularis (Weichselbaum-Jaeger), *Jahrb. f. Kinderheilk.*, 1896, xliii, 1; Zur Aetiologie und Diagnose der epidemischen Cerebrospinalmeningitis, *Deutsch. med. Wchnschr.*, 1896, xxii, 423; Noch einmal der Meningokokkus intracellularis, *Jahrb. f. Kinderheilk.*, 1902, lvi, 359.
- Hirsch, A., Handbuch der historisch-geographischen Pathologie, 2d edition, Stuttgart, 1886, iii, 379.
- Holt, L. E., The Treatment of Cerebrospinal Meningitis by the Serum of Flexner and Jobling, *Brit. Med. Jour.*, 1908, ii, 1336.
- Jobling, J. W., Standardization of the Antimeningitis Serum, *Jour. Exper. Med.*, 1909, xi, 614.
- Ker, C. B., The Treatment of Cerebro-Spinal Meningitis with Flexner's Serum, *Edinburgh Med. Jour.*, 1908, i, 306.
- Koplik, H., The Serum Treatment and the Prognosis, under Various Forms of Therapy, of Cerebrospinal Fever, *Med. Rec.*, 1908, lxxiv, 557.
- Levy, E., Erfahrungen mit Kolle-Wassermannschem Meningokokkenheilserum bei 23 Genickstarrekranken, *Klin. Jahrb.*, 1908, xviii, 317; Serumbehandlung der epidemischen Genickstarre, Jena, 1911; Die Behandlung der epidemischen Genickstarre durch Seruminjectionen in die Seitenventrikel. Bericht über einen geheilten Fall, *Arch. f. Kinderheilk.*, 1912, lix, 72.
- Louisiana State Board of Health Quarterly Bulletin, 1912, iii, No. 2.
- Netter, A., Traitement de la méningite cérébro-spinale, Association française de pediatrie, Congrès de 1910, Paris, 1911.
- Netter, A., and Debré, R., La méningite cérébro-spinale, Paris, 1911.
- Rieger, Die übertragbare Genickstarre im Kreise Brieg im Jahre 1905 und ihre Bekämpfung, *Klin. Jahrb.*, 1906, xv, 321.
- Robb, A. G., Some Observations on the Recent Outbreak of Cerebro-Spinal Fever in Belfast, *Brit. Med. Jour.*, 1907, ii, 1129; The Treatment of Epidemic Cerebro-Spinal Fever by Intraspinal Injections of Flexner and Jobling's Antimeningitis Serum, *idem.*, 1908, i, 382.
- Schepelmann, E., Genickstarre und Heilserum, *Wien. klin. Wchnschr.*, 1911, xxiv, 118.
- Schneider, Die übertragbare Genickstarre im Regierungsbezirk Breslau im Jahre 1905 und ihre Bekämpfung, *Klin. Jahrb.*, 1906, xv, 299.
- Sophian, A., A New Method for Controlling the Administration of Serum in Epidemic Meningitis, *Jour. Am. Med. Assn.*, 1912, lviii, 843; Epidemic Cerebrospinal Meningitis, St. Louis, 1913.
- Strümpell, A., Zur Pathologie und pathologischen Anatomie der epidemischen Cerebrospinalmeningitis, *Deutsch. Arch. f. klin. Med.*, 1882, xxx, 500.
- von Ziemssen, H., Meningitis cerebrospinalis epidemica, Handbuch der speciellen Pathologie und Therapie, Leipzig, 1878, §. ii, 639.

## PARALYSIS IN A DOG, SIMULATING POLIOMYELITIS.\*

BY SIMON FLEXNER, M.D., AND PAUL F. CLARK, PH.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

PLATES 103 TO 107.

The specimen about to be described<sup>1</sup> consisted of the spinal cord and intervertebral ganglia of a paralyzed dog removed from the recently chloroformed animal. Its history in brief is as follows:

The animal, a brindle Boston bull between four and five years old, was noted to have difficulty in swallowing; the mucous membrane of the throat was not red. The next day the left fore leg was lame and the animal was suspected of developing rabies. There was little fever, if any. The brain functions were normal. On the fourth day the left fore leg was completely flaccid; the head drooped and was raised with difficulty. The angles of the mouth drooped also. The paralysis progressed somewhat further in the muscles of the neck, and the right leg showed indefinite involvement. The dog was chloroformed on December 27 and the vertebral column was removed and brought to the Rockefeller Institute where the spinal cord and ganglia were taken out aseptically.

Dr. Zucker expressed the opinion that the paralysis in the dog resembled the paralysis of poliomyelitis in man and differed from the common distemper paralysis. This opinion is supported by the autopsy findings and microscopical examination.

The lesions in the spinal cord were obvious on gross examination and consisted of swollen, softened, and hemorrhagic foci in the cervical, and to a less extent in the lumbar region. Portions of the spinal cord were placed in sterile 50 per cent. glycerin, and on January 7 two puppies and two *Macacus rhesus* monkeys were inoculated with an emulsion prepared from the affected parts of the cord. One each of the dogs and monkeys was inoculated respectively intracerebrally and into the sciatic nerve and peritoneum. The inoculations were made under ether anesthesia and produced no result.

\* Received for publication, March 1, 1913.

<sup>1</sup> We are indebted to Dr. F. A. Zucker, of Elizabeth, New Jersey, for this interesting specimen.

The inoculated animals remained under observation for several months and developed no symptoms of paralysis.

The failure of the inoculations centers the direct interest upon the pathological changes in the spinal cord and intervertebral ganglia. They are pronounced. No part of the cord is free from changes and the most pronounced lesions are present in the cervical and lumbar enlargements. The lesions are not diffuse but they affect chiefly the anterior, and to a less extent the posterior gray matter. The white matter has escaped except to the extent that the contained blood vessels are often affected. The lesions consist of (*a*) perivascular infiltrations, (*b*) hemorrhage, (*c*) edema, infiltration, and necrosis of the ground substance followed by an invasion of large phagocytic cells (compound granular corpuscles), and (*d*) necrosis of ganglion cells which become, at times, replaced by small round cells. The lesions in the intervertebral ganglia consist of perivascular infiltration and accumulation of round cells about the ganglion cells, which rarely are degenerated. The perivascular infiltration is present in the nerve roots, but is less marked there than in the ganglia or spinal cord. The most pronounced lesions are about the blood vessels within the spinal cord, although vessels in the meninges not infrequently show marked infiltrations (figure 1). The largest arteries and veins tend not to be affected and the infiltration is most apparent about the intramedullary vessels at the margin of and within the gray matter (figure 2). The largest accumulations of cells are anterior, possibly because the vessels are of larger caliber, but arterioles and venules show some degree of cellular invasion. From the vessels the ground substance is invaded irregularly, and in many places the invading cells have surrounded ganglion cells showing more or less degeneration (figure 3).

The hemorrhages are small and confined to the immediate locality of blood vessels. But the edema extends at times throughout the gray matter. As a rule, the anterior horns are affected disproportionately. The rarefied ground substance stains lightly and is poor in cells except about the vessels and infiltrated ganglion cells. Focal necrosis of the gray matter involving ground substance, vessels, and nerve cells was confined to the cervical and lumbar enlargements. In the former it corresponded with the paralysis of the fore leg and

neck observed during life, and in the latter it was less extensive although still taking in a large fraction of the affected anterior horn. Very little of the original tissues remained except the coarser framework which now supported large numbers of phagocytic cells (figure 4).

Degeneration of the nerve cells occurred at all levels, but was most pronounced in the cervical and then in the lumbar region. The degenerated cells lacked protoplasmic processes and tigroid substance, were homogeneous and acidophilic and often shrunken. All stages of the degenerative process could be followed in different sections. About these nerve cells lymphoid cells accumulated, but rarely, if ever, invaded, although frequently taking the place of, the degenerated nerve cells. Neurophagocytosis was absent.

The infiltrating cells about the blood vessels and within the gray substance, excluding the phagocytic cells, were of the mononuclear character. They have the size and general appearance of cells of the lymphoid type (figure 5). Polynuclear cells appear to have been wholly absent even from the larger necrotic foci.

The lesions of the intervertebral ganglia are chiefly perivascular. The cellular infiltration is identical with that of the spinal cord except that the degree is less (figure 6). From the vessels the neighboring framework is invaded and the ganglion cells are surrounded. Rarely the pericellular infiltration is great enough to cause degeneration of such a cell (figure 7). Within the nerve roots the blood vessels show a similar, if slighter, grade of cellular infiltration (figure 8).

There is undoubted similarity in the pathological condition described with the lesions occurring in the spinal cord and intervertebral ganglia in poliomyelitis. The lesions in the cord and ganglia of the dog are, however, not identical with those of poliomyelitis in man or the monkey. The perivascular infiltration and the edema, hemorrhage, and necrosis of the gray substance of the cord are in close agreement, but the degeneration of the nerve cells is different. In poliomyelitis the necrotic nerve cells disappear less by a process of autolysis, but are removed more actively by phagocytes which may account for the distinct difference in appearances presented by the degenerating cells in the two conditions.

However, it is obviously improper to generalize from a single example, especially since the resemblances between the lesions in the dog and those characteristic of poliomyelitis are more numerous than the divergences. It would also be unsafe to conclude from the negative inoculations that the paralytic condition in the dog is of non-communicable character. The lesion in the spinal cord may well not have been as recent as the clinical history suggests, so that the most favorable period for the transmission of the disease may have passed at the time the inoculations were made. This important point, as well as the fact of the precise nature of the malady causing the lesions and the paralysis, still call for elucidation.

There is a well defined nervous disease of large domestic animals with which the condition of the dog bears some resemblance. In Borna's disease of horses, an extensive perivascular, mononuclear cellular infiltration occurs leading to paralysis and death. The lesions occur invariably in the brain and inconstantly in the spinal cord. From the vessels the ground substance of the nervous tissues may become infiltrated with cells, but the nerve cells show inconspicuous changes and, apparently, never severe degeneration or necrosis. The central nervous organs of man in sleeping sickness show a similar high degree of perivascular mononuclear infiltration most marked in the brain. Whether in this dog the brain was affected cannot be known as it was not submitted for examination.

#### CONCLUSION.

A pet dog developed a form of paralysis simulating the paralysis of epidemic poliomyelitis. The autopsy showed changes in the spinal cord and intervertebral ganglia, resembling, but not identical with, the lesions of poliomyelitis in man. Inoculation of the affected spinal cord into the nervous system of young dogs and *Macacus rhesus* monkeys was not followed by the production of paralysis or other obvious symptom of disease.

#### BIBLIOGRAPHY.

- Chiari, H., Demonstration eines Gehirns von Schlafkrankheit, *Atti d. Cong. internas. dei patologi*, 1911, Torino, 1912, i, 230.  
Flexner, S., and Lewis, P. A., Experimental Epidemic Poliomyelitis in Monkeys, *Jour. Exper. Med.*, 1910, xii, 227.



- Harbitz, F., and Scheel, O., Pathologisch-Anatomische Untersuchungen über akute Poliomyelitis und verwandte Krankheiten, Christiania, 1907.
- Joest, E., and Degen, K., Untersuchungen über die pathologische Histologie, Pathogenese und postmortale Diagnose der seuchenhaften Gehirn-Rückenmarksentzündung (Bornasche Krankheit) des Pferdes, *Ztschr. f. Infektionskrankh. parasit. Krankh. u. Hyg. d. Haustiere*, 1911, ix, 1.
- Mott, F. W., The Comparative Neuropathology of Trypanosome and Spirochæte Infections with a Résumé of our Knowledge of Human Trypanosomiasis, *Proc. Roy. Soc.*, 1910-11, iv, Path. Sec., 1.
- Wickman, I., Beiträge zur Kenntnis der Heine-Medinschen Krankheit, Berlin, 1907.

## EXPLANATION OF PLATES.

## PLATE 103.

FIG. 1. Spinal cord. Section showing lymphoid cell infiltration about vessels in the extra- and intramedullary meninges.  $\times 98$ .

## PLATE 104.

FIG. 2. Spinal cord. Section showing extensive lymphoid cell infiltration of the blood vessels at the margin of the anterior horn and also mononuclear cellular collection about nerve cells within the gray matter.  $\times 81$ .

## PLATE 105.

FIG. 3. Spinal cord. Lymphoid cell infiltration about the ganglion cell in the anterior horn; the nerve cell shows degeneration.  $\times 330$ .

FIG. 4. Spinal cord. Phagocytic cells within the degenerated gray matter of the anterior horn.  $\times 330$ .

## PLATE 106.

FIG. 5. Spinal cord. Lymphoid cell infiltration about a large vessel in the white matter.  $\times 144$ .

FIG. 6. Intervertebral ganglion. Lymphoid cell infiltration about a small vessel.  $\times 330$ .

## PLATE 107.

FIG. 7. Intervertebral ganglion. Lymphoid cell infiltration about degenerating ganglion cells.  $\times 330$ .

FIG. 8. Nerve root. Lymphoid cell infiltration about small vessels.  $\times 98$ .



FIG. 1.

(Flexner and Clark: Paralysis in a Dog Simulating Poliomyelitis.)



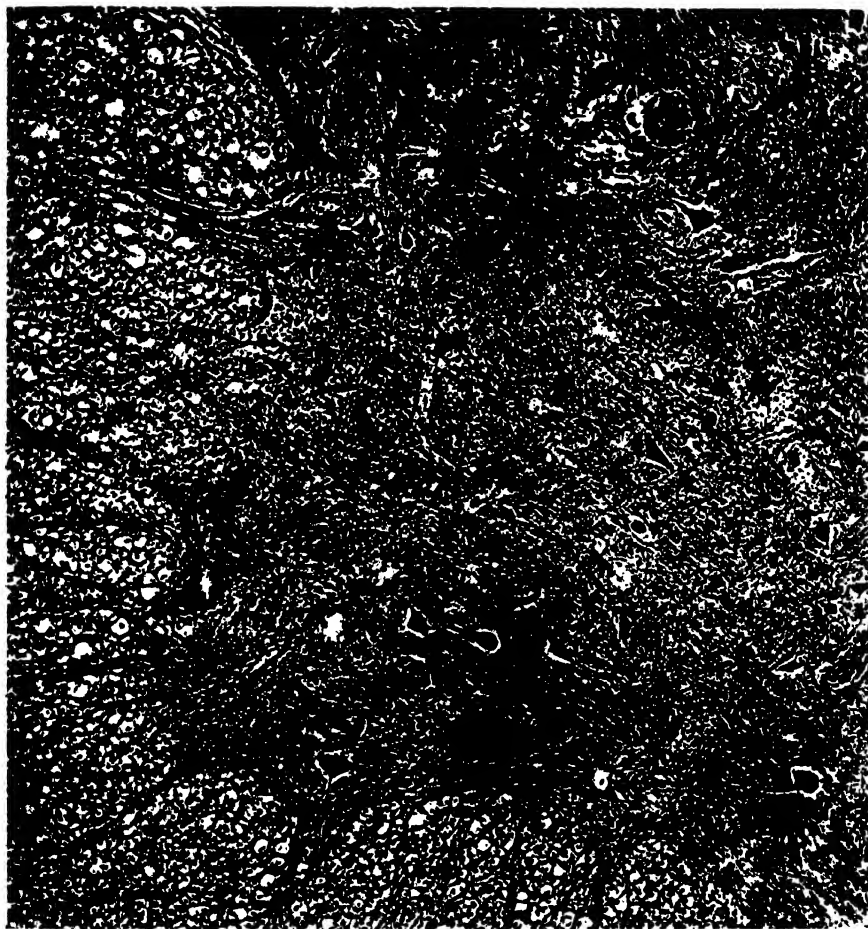


FIG. 2.

(Flexner and Clark: Paralysis in a Dog Simulating Poliomyelitis.)



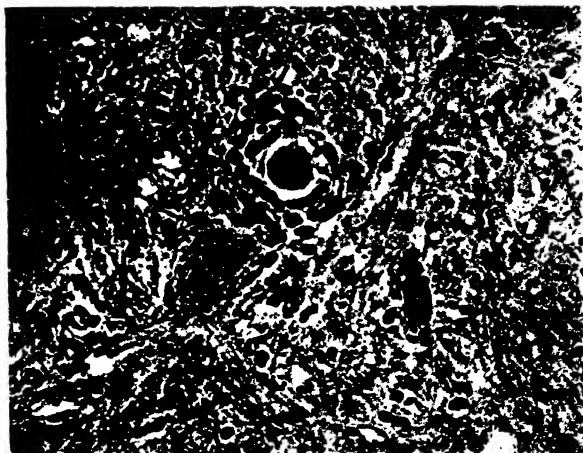


FIG. 3.

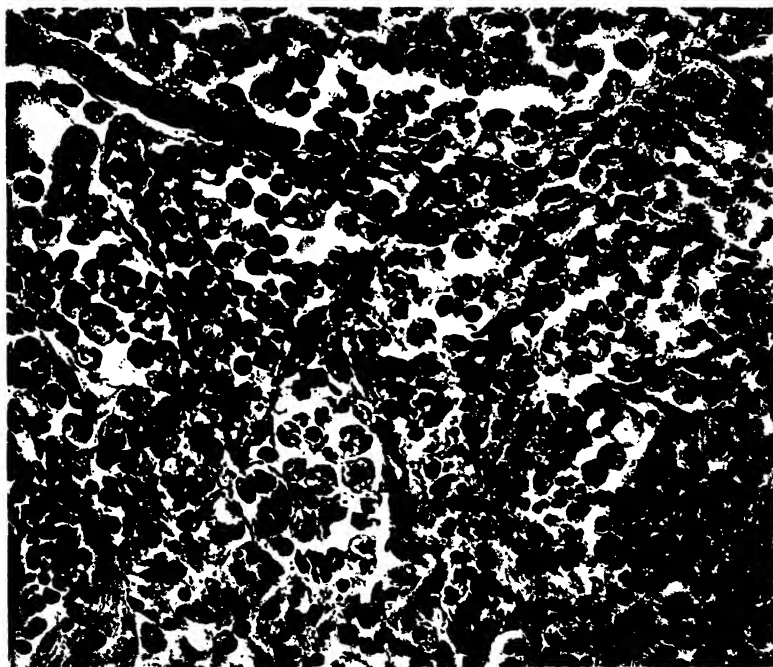


FIG. 4.

(Flexner and Clark: Paralysis in a Dog Simulating Poliomyelitis.)



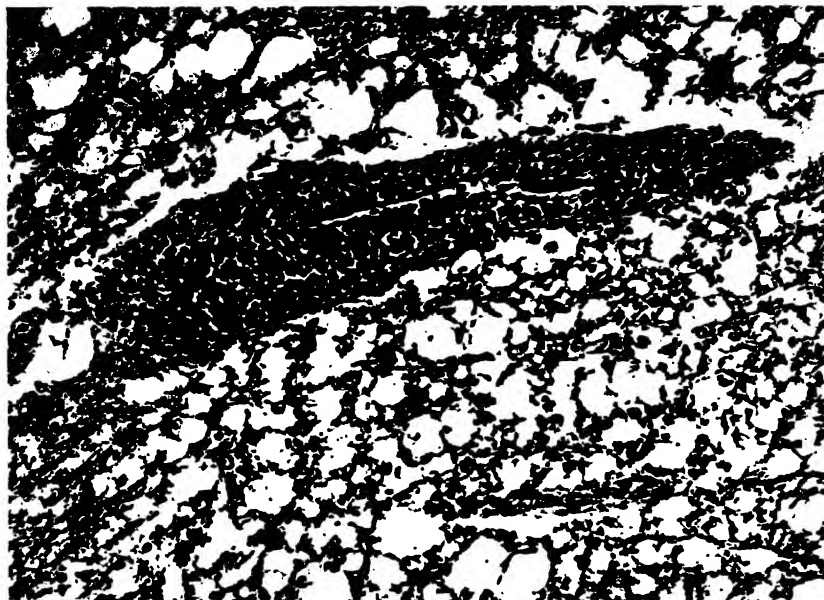


FIG. 5.

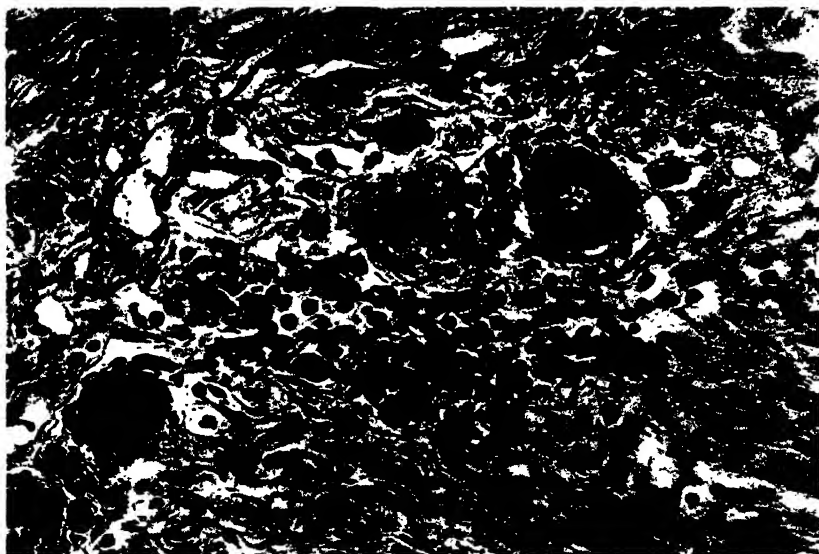


FIG. 6.

(Flexner and Clark: Paralysis in a Dog Simulating Poliomyelitis.)





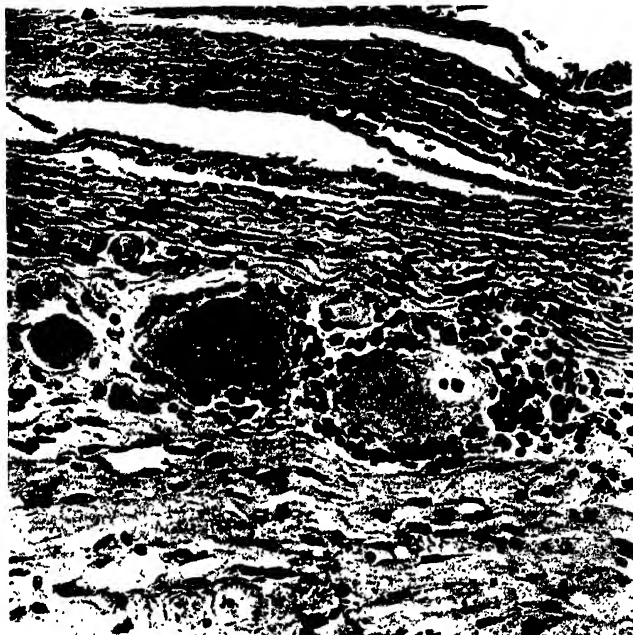


FIG. 7.



FIG. 8.

(Flexner and Clark: Paralysis in a Dog Simulating Poliomyelitis.)



## THE ACTION OF PNEUMOCOCCUS ON BLOOD.\*

By E. E. BUTTERFIELD, M.D., AND F. W. PEABODY, M.D.

(From the Laboratories and Hospital of The Rockefeller Institute for Medical Research, New York.)

During the winter of 1911 one of us (Peabody) made the observation that in some cases of lobar pneumonia the oxygen capacity of the blood is reduced below the normal level without a corresponding diminution of the hemoglobin content (Sahli hemoglobinometer). Several possibilities suggested themselves: (1) the presence of reducing substances in the serum; (2) a shift of the equilibrium between oxygen and hemoglobin, due possibly to an increase in the concentration of H<sup>+</sup> ions in the serum; (3) the formation of some derivative of hemoglobin which does not combine with oxygen. Experiments with washed corpuscles from these patients showed that a reduction of the oxygen capacity still persisted after removal of the serum. This result eliminated from consideration the presence of reducing bodies and a shift of equilibrium due to some constituent of the serum, and led us to regard the diminution of the oxygen capacity as the manifestation of some irreversible constitutive change in the oxyhemoglobin within the red cells. It was soon found that a similar drop in the oxygen capacity of the blood occurred in guinea pigs and rabbits with pneumococcus infection, and, furthermore, that the action of pneumococcus cultures on washed rabbit corpuscles *in vitro* also produced constantly a definite drop in the oxygen capacity and light absorption. This gave us a convenient means of studying the phenomenon under varied and controllable conditions, with the object in view to determine, if possible, what derivative of hemoglobin was formed in the process.

The method decided upon was to take equal volumes of washed rabbit corpuscles and to add to one portion, which was to serve as

\* Received for publication, March 10, 1913.

a control, a known volume of 0.9 per cent. sodium chloride,<sup>1</sup> and then to add the same volume of a bouillon culture of virulent pneumococcus to the other portion of washed rabbit corpuscles. Both preparations were kept at 37° C., usually for eighteen hours, after which they were laked with equal volumes of distilled water and centrifuged. In the clear supernatant fluid of control and pneumococcus preparation the oxygen capacity (Haldane-Barcroft method) and the light absorption (spectrophotometer of Martens, König, and Grünbaum) were then directly determined.<sup>2</sup> Both preparations were vigorously shaken with air before any measurements were made.

There exists between the light absorption in two regions of the spectrum and varying concentrations of oxyhemoglobin in the presence of some other derivative of hemoglobin a mathematical relation. We shall not go into the derivation of the formula here, but merely state the relations in their final form. For the relation between the light absorption of a mixture and the relative concentration of oxyhemoglobin (or of the second derivative), the sum of both concentrations being taken equal to 1, one finds:

$$\frac{\log \frac{I_1}{I_1'}}{\log \frac{I_2}{I_2'}} = \frac{(\beta_1 - \alpha_1)x + \alpha_1}{(\beta_2 - \alpha_2)x + \alpha_2} \quad (1)$$

$I_1$  and  $I_1'$  are, respectively, the intensities of the light entering and leaving the solution at a given wave length in the spectrum,  $\alpha_1$  is the absorption constant of oxyhemoglobin, and  $\beta_1$  the absorption constant of the second derivative at the same wave length.  $I_2$ ,  $I_2'$ ,  $\alpha_2$ , and  $\beta_2$  are the corresponding quantities at another wave length in the spectrum,  $x$  is the relative concentration of the second derivative, and  $(1 - x)$  would be the relative concentration of oxyhemoglobin.

For the relation between the absolute concentration of oxyhemoglobin in the presence of another substance with optical constants

<sup>1</sup> Sterile bouillon was also used as a diluent for the control in some experiments, without appreciably affecting the results.

<sup>2</sup> For measurement of the light absorption in concentrated solutions of oxyhemoglobin see Butterfield, E. E., *Ztschr. f. physiol. Chem.*, 1912, lxxix, 439.

$\beta_1$  and  $\beta_2$  and the light absorption at two different wave lengths, one finds:

$$c_1 = \frac{2.30 \left( \beta_2 \log \frac{I_1}{I'_1} - \beta_1 \log \frac{I_2}{I'_2} \right)}{l(\alpha_1 \beta_2 - \alpha_2 \beta_1)}. \quad (2)$$

Here  $c_1$  is the absolute concentration of oxyhemoglobin and  $l$  linear thickness of the absorbing layer. The other symbols have the same significance as in formula (1).

To apply these formulas to the present problem it would be necessary to know the optical constants of oxyhemoglobin and those of the second derivative of hemoglobin. But the second derivative is one of our unknowns. It would, therefore, be necessary to take the measured values for the light absorption and try out a series of analogous formulas in all of which the optical constants of oxyhemoglobin figure, but in each of which a different set of constants corresponding to the different derivatives of hemoglobin occurs. If a formula is found which gives, for the change in the oxyhemoglobin concentration of the pneumococcus preparation as compared with the control, values agreeing with those for the change in the oxygen capacity, the second derivative is then identified with a high degree of probability. Our course was fortunately much shortened by the observation that when pneumococcus culture is incubated with laked blood there appears spectroscopically an absorption band in red. When washed corpuscles are used the process rarely progresses to such an extent that an absorption band in red becomes visible. This narrowed the choice of the second derivative down to methemoglobin or hematin. Furthermore, Grüter<sup>3</sup> has made excellent spectroscopic observations on blood dropped into pneumococcus cultures. From the appearance of the absorption band in red and from the spectroscopic changes on reduction he reached the conclusion that some substance in a pneumococcus culture converts oxyhemoglobin into methemoglobin.<sup>4</sup> Therefore

<sup>3</sup> Grüter, W., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1900, 1, 241.

<sup>4</sup> This method is not entirely free from objection, because the spectrum of the reduction product of hematin, hemochromogen, in relatively small quantities, would be masked by the diffuse absorption of reduced hemoglobin. It would be thus impossible to distinguish hematin from methemoglobin in the presence of oxyhemoglobin except in the case of relatively large amounts of hematin.

we were led at first to use the optical constants of methemoglobin together with those of oxyhemoglobin in the formula for the light absorption as a function of the concentration. Accordingly, crystalline oxyhemoglobin and methemoglobin were prepared from ox blood and the light absorption was determined in dialyzed solutions at the wave lengths  $577$ ,  $579\mu$  (double line, mercury lamp),  $546\mu$  and  $436\mu$ . For the present work the constants at  $577$ ,  $579\mu$  and  $546\mu$  were used. The values are for oxyhemoglobin  $\alpha_1=21.00$  and  $\alpha_2=18.42$ , for methemoglobin  $\beta_1=5.18$  and  $\beta_2=7.31$ .

The first table represents data collected to show the degree of agreement between the gasometric and photometric measurements for widely varying values of the relative concentration,  $x$ . In table I the percentage concentration  $100x$  is used.

TABLE I.

	Control.		Pneumococcus preparation.		Per cent. diminution of oxygen capacity.	Per cent. diminution of oxyhemoglobin concentration.	Nature of experiment.
	Oxygen capacity.	$\frac{\log \frac{I_1}{I_1'}}{\log \frac{I_2}{I_2'}}$	Oxygen capacity.	$\frac{\log \frac{I_1}{I_1'}}{\log \frac{I_2}{I_2'}}$			
I	3.26	1.14	2.93	1.12	10	11	Washed rabbit corpuscles + bouillon culture pneumococcus + sodium hydroxide.
II	3.40	1.14	2.87	1.11	16	16	Washed rabbit corpuscles + bouillon culture pneumococcus.
III	16.96	1.15	12.98	1.10	24	21	Washed rabbit corpuscles + bouillon culture pneumococcus.
IV	5.92	1.14	4.36	1.08	26	29	Washed rabbit corpuscles + bouillon culture pneumococcus.
V	3.65	1.14	1.09	0.96	70	64	Laked rabbit blood + bouillon culture pneumococcus.
VI	3.40	1.14	0.37	0.79	89	91	Laked rabbit blood + bouillon culture pneumococcus.

The agreement is excellent in most cases, but can only be realized in the case that the dilutions are so accurate that control and pneumococcus preparation have the same initial concentration of oxyhemoglobin. The difficulty in obtaining this lies in the sedi-

mentation of the red cells. Formula (2), which gives the relation between the absolute concentration of oxyhemoglobin and the change in the light absorption, is independent of dilution. We give two examples of the use of this formula.

TABLE II.

*Relation between Oxygen Capacity and Absolute Concentration of Oxyhemoglobin.*

	$\log \frac{I_1}{I_1'}$	$\log \frac{I_2}{I_2'}$	$c_1$ in gm. per 100 c.c.	Oxygen ca- pacity, volume per cent.	Per cent. diminution in	
					$c_1$	Oxygen capacity.
I { Control. ....	0.523	0.457	2.72	3.84	14	15
Culture. ....	0.466	0.418	2.34	3.27		
II { Control. ....	0.437	0.385	2.30	3.26	32	32
Autolysate. ....	0.321	0.292	1.57	2.23		
Thickness of light-absorbing layer = 0.021 cm.						

Here it is seen that the agreement between gasometric and optical results is even better than in the first series. It will be noticed from the second experiment of the second series that the autolysate of pneumococcus cultures, which in this case was filtered and sterile, also produces the same effect on blood as does the growing pneumococcus.

The identification of the substance or substances in the pneumococcus cultures, which induces the formation of methemoglobin, is a problem in itself and one into which we do not care to enter here. However, in view of the well known acid production by the pneumococcus it seemed worth while to try the effect of acid on the oxygen capacity and light absorption of the blood, even though our pneumococcus-blood mixtures never showed a definite acid reaction to litmus. Table III gives the result of some experiments in this direction.

It is evident from the results that amounts of acid, which in proportion to the blood volume would be considerable for the living organism, have but little effect on the oxygen-combining power of the blood, and this not at all in the direction of a diminution of the oxygen capacity.



TABLE III.

*Acid Series.*

	Oxyhemoglobin concentration.	Oxygen capacity.	Oxygen capacity Oxyhemoglobin concentration.	Quantity of n/20 hydrochloric acid added to 4.5 c.c. of washed rabbit corpuscles, in c.c.
I	4.27	5.97	1.40	0.0
II	4.47	6.25	1.40	0.5
III	4.48	6.37	1.42	1.0
IV	4.40	6.35	1.44	2.0

## CONCLUSION.

The reduction of the oxygen capacity which occurs after incubating pneumococcus cultures with washed rabbit corpuscles is due to the formation of methemoglobin (or some derivative of hemoglobin with identical optical constants for three regions in the spectrum). The substance which induces the change is also present in the sterile filtrate of autolyzed cultures. By analogy we feel justified in concluding that the mechanism of the reduction of the oxygen capacity in human lobar pneumonia is at least in part of the same nature. To determine the frequency and intensity of the phenomenon in lobar pneumonia, and thereby to establish its clinical significance, is the next step and a problem upon which we are now engaged.

## THE COLOR INDEX AND COLOR OF THE RED BLOOD CORPUSCLES.

By E. E. BUTTERFIELD.

*(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)*

The present study is based largely on material observed several years ago in Munich in the clinic of Prof. Friedrich Müller. The object of the study was to determine by means of exact methods, (1) the existence of a high color index in pernicious anemia, (2) the magnitude of the elevation of the color index, and (3) the explanation of the phenomenon.

The calibration of the pipette and the dimensions of the counting chamber used for the erythrocyte counts were checked by special methods. The hemoglobin determinations were made spectrophotometrically (spectrophotometer of König, Martens and Grünbaum, Nernst filament as light source). The manner in which the hemoglobin concentration may be calculated from a measurement of the light absorption follows from the equation for the diminution in the intensity of homogeneous light on traversing a planparallel layer of a colored solution:

$$I' = Ie^{-kcl}, \quad (1)$$

in which  $I$  = initial intensity,  $I'$  = final intensity,  $k$  = a constant,  $l$  = linear thickness of the absorbing layer, and  $c$  = the concentration of the colored substance.  $I'/I$  can be measured with spectrophotometer. When this has been done with a solution of pure oxyhemoglobin of known concentration in an absorption tube of known length  $k$  can then be calculated. After the value of  $k$  has been determined for pure oxyhemoglobin from human blood the concentration of hemoglobin in laked human blood can be derived from a measurement of  $I'/I$ . Oxyhemoglobin from ox blood may

be conveniently substituted for human hemoglobin in the determination of  $k$  since it was shown in Tübingen that the constant has the same value for both hemoglobins. In the present study homogeneous light was not employed, but the measurements were made in a very narrow interval in the spectrum (about  $4\mu\mu$ ). This is not accompanied by an appreciable error as long as the concentration and linear thickness are kept within narrow limits. The measurements were always made in two regions in the spectrum, (1) at the maximum in green of the second absorption band of oxyhemoglobin, and (2) at the minimum in yellowish green between the two absorption bands of oxyhemoglobin. The reason for this follows on combining the two equations for these measurements. If  $I_1' = I_1 e^{-k_1 l_0}$  represent the conditions in the first region and  $I_2' = I_2 e^{-k_2 l_0}$  those in the second region, one would have after logarithmating and dividing,

$$\frac{\log \frac{I_1}{I_1'}}{\log \frac{I_2}{I_2'}} = \frac{k_1}{k_2} = K. \quad (2)$$

Therefore

$$\frac{\log \frac{I_1}{I_1'}}{\log \frac{I_2}{I_2'}}$$

remains constant independent of concentration and linear thickness. If

$$\frac{\log \frac{I_1}{I_1'}}{\log \frac{I_2}{I_2'}}$$

varies then some other substance with constants different from  $k_1$  and  $k_2$  must also be present in the solution. For pure oxyhemoglobin  $k_1 = 19.36$  and  $k_2 = 11.66$ . As concentration grams in 100 c.c. is used. The blood was taken in all cases from an arm vein and immediately defibrinated. Table I gives the result of

determinations made chiefly on individuals in apparent good health and on patients with pernicious anemia.

TABLE I.  
*Normal Blood.*

	Specific gravity.	Sahli reading.	Erythrocytes. Millions per cu. mm.	$\log \frac{I_1}{I_2}$ $\log \frac{I_1}{I_2}$	Hemoglobin content of blood. Grams per 100 c.c.	Quantity of hemoglobin in average erythrocyte. Grams $\times 10^{-11}$ .	Color index.
Men, 1	1.057	95	4.29	1.66	15.4	3.6	1.1
2	1.055	77	4.19	1.65	14.3	3.4	1.0
3	1.062	100	5.19	1.67	17.4	3.2	0.9
4	1.061	97	5.46	1.68	17.8	3.3	1.0
5	1.056	87	4.57	1.64	15.3	3.4	1.0
6	1.066	105	5.79	1.65	19.3	3.3	1.0
7	1.060	93	4.74	1.65	16.9	3.6	1.1
Averages. ....	1.059	93	4.92	1.66	16.6	3.4	1.0
Women, 1	1.057	83	4.42	1.64	15.8	3.6	1.1
2	1.058	88	5.11	1.64	15.6	3.1	1.0
3	1.053	77	4.44	1.67	13.7	3.1	1.0
4	1.061	97	5.11	1.64	17.0	3.3	1.0
5	1.055	85	4.46	1.64	13.7	3.1	1.0
6	1.056	90	4.95	1.65	15.2	3.1	1.0
Averages. ....	1.057	87	4.75	1.65	15.2	3.2	1.0

*Pathological Cases.*

Pernicious anemia I. ....	1.040	21	0.74	1.65	3.47	4.7	1.5
Pernicious anemia II. ....	1.035	23	0.87	1.65	3.79	4.4	1.3
Secondary anemia. ....	—	—	2.43	1.63	5.59	2.3	0.7
Polycythemia. ....	1.075	—	—	1.66	23.9	—	—

It will be seen first that the average hemoglobin content of defibrinated normal blood is considerably higher than the orthodox 14 grams in 100 c.c. Secondly, the value for the amount of hemoglobin per corpuscle in normal blood is remarkably constant. This may be taken as a measure of the color index, and the color index may be conveniently defined as the quantity of hemoglobin per corpuscle multiplied by that factor which renders the quantity of hemoglobin per normal corpuscle = 1. In pernicious anemia there is a marked increase in the quantity of hemoglobin per corpuscle.

That this is an actual increase and not a simulated effect due to the presence of some substance with a greater light absorption than oxyhemoglobin is shown by the constant value for

$$\frac{\log \frac{I_1}{I_1'}}{\log \frac{I_2}{I_2'}}$$

in normal blood and in the blood of pernicious anemia.

Closely related to the color index is the actual color of the red blood corpuscles. It is well known that the color of erythrocytes in single layer viewed microscopically in transmitted light is yellowish green. The blood itself is deep red in color, and the reddish tinge becomes noticeable microscopically when several superposed corpuscles are viewed in transmitted light. The explanation of this phenomenon becomes apparent, I think, when one studies the spectrum of a single layer of corpuscles as compared with several superposed layers. The absorption curve of oxyhemoglobin presents a minimum in yellowish green ( $560\mu\mu$ ) and a region of least absorption in red ( $650\mu\mu$ – $660\mu\mu$ ). In the oxyhemoglobin spectrum  $k_{gr}$  is much greater than  $k_r$ , consequently on increasing the thickness of the absorbing layer the intensity of the transmitted light diminishes much more rapidly in yellowish green than in red. If we regard only these two regions in the spectrum we would have for the intensity of the transmitted light in yellowish green  $I_{gr}' = I_{gr}e^{-k_{gr}l}$  and in red  $I_r' = I_re^{-k_rl}$ . For  $I_{gr}e^{-k_{gr}l} > I_re^{-k_rl}$  a color change would be expected. This is known as the principle of dichromatism. Whether a color change occurs or not with the same light source would depend on the values of  $k_{gr}$  and  $k_r$  and on  $l$ . The necessary conditions  $I_{gr} > I_r$ ,  $k_{gr} > k_r$  for oxyhemoglobin and  $l$  sufficiently small are all realized in the case of red blood corpuscles viewed in daylight. This formulation is only a very rough approximation. The exact formulation would require integration of the intensities over the whole spectrum, and this cannot be done at present as long as it is not known what functions  $I$  and  $k$  are of  $\lambda$  (the wave-length). However, several observations furnish strong support for the view

that the color change of erythrocytes in layers of varying thickness is in accordance with the principle of dichromatism. It is possible to construct a thin wedge of solid oxyhemoglobin and observe the thickness at which the color change occurs. In such a wedge at a thickness of  $1.3\mu$  and less the color is identical with that of a single layer of red blood corpuscles. Above  $1.3\mu$  a distinct reddish tinge is noticeable, increasing with the thickness of the wedge to a deep pure red. In this experiment the color is the same in parallel or convergent light. This rules out the influence of the stroma and the surface curvature on the color of the red blood corpuscles. Finally, small (microscopic) crystals of oxyhemoglobin (second crystallization) are of the same color as the red blood corpuscles, while larger (*i. e.*, thicker) crystals are bright red.

## PNEUMONIC LESIONS MADE BY INTRABRONCHIAL INSUFFLATION OF NON-VIRULENT PNEUMOCOCCI.\*

By MARTHA WOLLSTEIN, M.D., AND S. J. MELTZER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

### INTRODUCTION.

In two series of investigations pneumonia has been produced experimentally in dogs by means of intrabronchial insufflation of virulent organisms. Lamar and Meltzer<sup>1</sup> produced lobar pneumonia in a large number of animals by insufflation of cultures of a highly virulent pneumococcus, and in a smaller number by the insufflation of *Streptococcus mucosus* and of Friedländer's pneumobacillus. We<sup>2</sup> produced lobular pneumonia in twenty dogs by the insufflation of cultures of streptococcus, and in eleven dogs by the insufflation of cultures of the influenza bacillus. These experimental results agree with the pathological experience with human beings; in lobar pneumonia it is the pneumococcus which is found in the exudate, and in bronchopneumonia the streptococcus is more often present. Both forms of experimental pneumonia have, of course, essential features in common; namely, there is in both an acute exudative inflammation, the exudate containing polymorphonuclear leucocytes, serum, and desquamated epithelial cells. Later large mononuclear phagocytes and cell debris also appear in both. But the pneumonias have also some features which sharply distinguish one from the other. We discussed these distinguishing features in our last paper.<sup>3</sup> For the purpose of our present paper it is well to enumerate them again. However, we wish to say expressly that these features have reference only to the experimental

\* Received for publication, December 10, 1912.

<sup>1</sup> Lamar, R. V., and Meltzer, S. J., *Jour. Exper. Med.*, 1912, xv, 133.

<sup>2</sup> Wollstein, M., and Meltzer, S. J., *Jour. Exper. Med.*, 1912, xvi, 126.

<sup>3</sup> Wollstein, M., and Meltzer, S. J., *loc. cit.*

pneumonias produced by the method of intrabronchial insufflation, and only as we know them at present.

1. In experimental lobar pneumonia there was a mortality of 16 per cent.; in experimental bronchopneumonia there was no mortality. We should, however, add that in this respect the two series of experiments were not sufficiently comparable to permit of a final conclusion. Not only was the number of experiments with the streptococcus only about one half as large as the number of those of Lamar and Meltzer with the pneumococcus, but most of the animals were killed at too early a stage after the inoculation to permit drawing a definite conclusion as to the actual mortality of this infection. For the present we have to be satisfied with the statement that in none of our uncomplicated cases of experimental bronchopneumonia was there a fatal tendency of the disease. Furthermore in the experiments with the pneumococcus the course seemed to depend upon the quantity of injected culture ("most of the deaths followed the injection of a large quantity of culture, and, conversely, the injection of a large quantity was usually fatal"),<sup>4</sup> while in our experiments with the streptococcus large doses did not seem to affect the final outcome. For instance, in an animal which received thirty cubic centimeters of culture and which was killed on the sixth day, the animal was not sicker than other animals that received fifteen or ten cubic centimeters of the culture, nor was the resolution of the exudate retarded.

2. In the pneumococcus inoculations a pneumococcus septicemia developed in all the fatal cases; no bacteremia occurred in the streptococcus cases.

3. In the pneumococcus lesions the consolidated part of the lung was always dense and air-free, *i. e.*, lobar in character, no matter how large or small the affected area was; in the lesions produced by the streptococcus, no matter how large the area or how intense the inflammatory process was, aerated lobules were always discernible among the solid foci; *i. e.*, the streptococcus lesion was lobular.

4. In pneumococcus pneumonia there was always a definite pleurisy present even in all the non-fatal cases, while it was practically absent in all the streptococcus infections.

<sup>4</sup>Lamar, R. V., and Meltzer, S. J., *loc. cit.*, p. 141.



5. In the pneumococcus lesions the cut surface was rather dry and granular; in the lesion produced by the streptococcus the cut surface was moist and smooth.

6. Fibrin was an important element in the exudate of the lesions brought about by the pneumococcus, while it played practically no part in the streptococcus pneumonia.

7. In the pneumococcus lesions the walls of the alveoli and bronchioles remained free from infiltration although their lumina may have been densely packed with exudate; on the other hand, in the lobular pneumonia produced by the streptococcus not only the walls of the alveoli and bronchioles but also the connective tissue septa and the adventitia of the blood vessels were the seat of marked infiltration.

8. The resolution of the pneumonic lesions set in and terminated earlier in the consolidations from streptococci than in those caused by pneumococci.

We may now add a further point of differentiation. In the course of further study we made many experiments with insufflation of virulent pneumococci. We found that in every case twenty-four hours after insufflation of a virulent pneumococcus this organism could be cultivated from the heart's blood, whereas three or four days later in the non-fatal cases the lungs as well as the blood were free from the pneumococcus. This is an interesting point and *probably has its analogy in clinical findings*. On the other hand, in pneumonia produced by the insufflation of streptococcus the blood remained free from the infecting organism. We may therefore state:

9. Insufflation of virulent pneumococci leads in all cases to bacteremia at the end of the first day after inoculation. The bacteremia disappears in the non-fatal cases, while after insufflation of streptococci no bacteremia develops.

From the above it seems that in general the inoculation of the streptococcus produced a milder effect than the pneumococcus. In discussing the results of both series of investigations, we said that since different organisms introduced in the same way and under conditions which are apparently the same produce distinctly different pneumonic lesions in animals of the same species, the con-

clusion presents itself that different types of pneumonia are produced by specifically different bacteria. We added, however, that further investigation may show that the differences in the nature of the lesions are due rather to the degree of virulence of the causative microorganism than to differences in the species, that even apparently virulent streptococci may nevertheless be less virulent than certain virulent pneumococci, and that such different lesions as were here obtained by different organisms may perhaps be obtained by organisms of the same species possessing different degrees of virulence.

This point has now been tested in a series of experiments on dogs with the intrabronchial insufflation of a non-virulent strain of pneumococcus. The non-virulent strain was obtained from Dr. Dochez of the Rockefeller Institute Hospital, by whom it was isolated from the blood of a patient with lobar pneumonia who recovered. The microorganism<sup>5</sup> had the morphological and cultural features of the pneumococcus, but failed to kill a mouse in doses less than one half cubic centimeter of a twenty-four hour broth culture when freshly isolated from the patient's blood. After cultivation in the laboratory it failed to kill in any dose.

#### EXPERIMENTAL DATA.

Twenty to twenty-four hour broth cultures of the avirulent pneumococcus were injected by intrabronchial insufflation into eighteen dogs, in doses varying from ten to thirty cubic centimeters. In three instances the dose of cocci was enriched three times; that is, it contained three times the number of organisms, as the result of centrifugalization of a large quantity of culture. The method of injection by intrabronchial insufflation has been described in detail by Lamar and Meltzer,<sup>6</sup> and consists essentially of the introduction of a tube through the mouth, larynx, and trachea deeply into a

<sup>5</sup> This strain of pneumococcus was soluble in rabbit bile, though it was avirulent for white mice, and, as will be shown later, it differed from the virulent pneumococcus in its effect on the lungs of dogs. This solubility of an avirulent pneumococcus is contrary to the statement of Neufeld and Haendel (Kolle, W., and Wasserman, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 1912, iv, 527) that avirulent strains are not dissolved in bile.

<sup>6</sup> Lamar, R. V., and Meltzer, S. J., *loc. cit.*

bronchus, the culture being introduced through the tube. Usually the tube slips into the right bronchus.

None of the dogs died as the result of the inoculation of these doses of avirulent pneumococci. All were killed at periods varying from one to seven days after the dose had been administered. The animals did not appear to be very ill, and only three showed a rise of temperature to  $41.1^{\circ}$  C., all three having received a dose of ten cubic centimeters. The animals receiving the enriched doses, as well as one dog to whom thirty cubic centimeters were given, had even less fever and no more severe local reaction than the dogs to whom the smaller dose had been administered.

*The Lesions in the Lungs.*—The gross appearance of the lesions in the lungs of the dogs varied according to the length of time elapsing between the injection and the killing of the animal; but in parallel stages the differences were very slight. The size of the dose influenced the course of the disease and the severity of the lesion but little, a point in marked contrast to the experience of Lamar and Meltzer with virulent pneumococci. There was no exudate on the surface of the pleura in any one of the eighteen cases, but a lack of luster was noted in three instances, one and two days after inoculation. In twenty-four hours after insufflation of a dose of ten or fifteen cubic centimeters of the avirulent organisms, the whole or part of one lobe was the seat of hepatization. In two cases the right posterior lobe, in two cases the left posterior lobe, and in one case the right anterior lobe was involved. In two cases the entire lobe was solid, in two others only two thirds of the lung was involved, and in one all but the superior anterior angle of the lobe was solid. The lobe was heavy, firm, but not much larger than its uninvolved neighbors. On section the hepatized lung was red, moist, and evenly consolidated; only blood could be expressed. From the bronchi frothy fluid ran, thin, not viscid. Aerated lobules were not seen within the solid areas, but at their periphery, the line of demarcation being irregular. Pneumococci were found in small numbers in smears made from the lung, and they grew sparingly in cultures; the heart blood was sterile. The anatomical diagnosis was that of an early, not severe lobar pneumonia in the stage of red hepatization, the condition of engorgement being still present in portions of the lobe.

In forty-eight hours the lesion had become more marked; hepatization was more advanced, though no larger area of lung was involved. Thus the left posterior lobe except its inferior border was solid in one case, three quarters of the right posterior in another, two thirds of the left anterior and the left posterior in another, and two thirds of the right posterior with a small area in the subcardiac lobe in still another animal. These lungs were more solid and heavy than those seen on the earlier day; on section they were mottled red and gray, smoothly consolidated, friable, and less moist. It was difficult to find pneumococci in smears, and none grew in cultures.

Microscopic examination after twenty-four hours showed that the alveoli were filled with polymorphonuclear leucocytes in large numbers, very few epithelial cells, and little fibrin. The capillaries in the alveolar walls were distended with blood, but the walls of the alveoli were not infiltrated with cells nor with fibrin. The congestion was most marked just beneath the pleura, and some of the most solidly packed areas were also there. The alveoli were not equally filled with exudate, some containing only fibrin and a few desquamated epithelial cells, others a few red cells in addition to these, and in others the leucocytes were most abundant, nuclear fragmentation being well under way. The smallest bronchi contained plugs of fibrin and leucocytes, while their walls showed leucocytic infiltration; the larger bronchi, on the other hand, were quite normal, showing neither desquamation of their epithelia nor infiltration of their walls; they were empty. Some medium sized bronchi contained masses of fibrin, leucocytes, and red cells quite detached from their lining cells, evidently sputum in the course of expectoration. Pneumococci were few in number in the alveoli and in the bronchi. Areas of leucocytic exudation about small veins were numerous.

In forty-eight hours the exudate was found to have become more leucocytic in character, the epithelial cells being few in number and the red blood cells only occasional. The amount of fibrin had not increased. The most solid areas were again found beneath the pleura. The bronchi showed the same lesions as in the earlier stage, the infiltration of their walls not being more marked or more general.

Five days after insufflation of a dose of ten cubic centimeters resolution was going on in a large portion of the right posterior lobe, though a small area of pneumonia still remained. In six days there were no solidly packed alveoli remaining, and resolution was almost complete.

A larger dose of twenty cubic centimeters had caused a lobar pneumonia still at its height on the third day, with an exudate consisting mostly of polynuclear leucocytes and with little fibrin. That the disease was still progressing on the third day was evidenced in the animal by the fact that while the right posterior lobe showed the lesion of red hepatization, the upper half of the left posterior lobe showed an earlier stage of engorgement and edema, with an exudation of serum, epithelial cells, red blood cells in small numbers, and less fibrin than was present in the later stage. This left posterior lobe was not swollen, but dark, firm, heavier than its aerated neighbor, and its section was moist, not crepitant, but not entirely airless.

The pneumonia caused by a dose of twenty cubic centimeters was quite resolved on the sixth and seventh days, leaving only a dark, congested, inelastic area to indicate grossly where the lesion had been. On microscopic examination there was evidence of phagocytosis of cellular fragments and the presence of granular, poorly staining debris in some alveoli.

In a dog that received thirty cubic centimeters the lungs presented on the third day a well marked but not intense lobar pneumonia (red hepatization) in the upper half of the right posterior lobe. The amount of fibrin present in the alveolar exudate was only moderate, leucocytes being very numerous.

An enriched dose, containing in fifteen cubic centimeters of broth the cocci grown in forty-five cubic centimeters, produced a lesion in twenty-four hours which occupied the right posterior lobe except its anterior border; the lobe was red, solid, not friable, evenly consolidated, moist, and without pleurisy. Its exudate consisted of polymorphonuclear leucocytes, few epithelial cells, and very little fibrin contained in alveoli whose walls were quite free from infiltration of any kind. The lesion was not more severe than one produced by a dose of fifteen cubic centimeters. Another

dog, after an enriched dose similar to the above, showed a resolving lobar pneumonia on the third day, about two thirds of the left posterior lobe having been involved. On microscopic examination the extent of the resolution was surprisingly far advanced, considering the size of the dose of cocci administered.

#### DISCUSSION.

The experiments established, in the first place, the fact that the insufflation of a culture of avirulent pneumococcus invariably caused the development of a pulmonary lesion which macroscopically had the appearance of a lobar pneumonia. The consolidated part was firm, solid, heavy, and contained no aerated lobules in its midst. The inflammation, however, was less intense than it usually is in the lesions of lobar pneumonia caused by virulent pneumococci, the progress was generally slower, and the resolution set in earlier. Furthermore, in none of the animals had the disease a fatal termination, and the increase of the quantity of the insufflated culture had not the same effect upon the course of the inflammatory process that it undoubtedly had in the experiments with the virulent pneumococcus. Still more important perhaps are the following differences. The exudate produced by the avirulent pneumococcus contained strikingly less fibrin than the exudates of the virulent pneumococci, the quantities of fibrin present being only slightly larger than in the streptococcus exudates. The pneumonia produced by the avirulent pneumococcus differed further from the pneumonia of the virulent pneumococcus by the complete absence of the organism from the blood at any time after the inoculation. Even the pulmonary lesion itself contained no more culturable organisms on the second day after the inoculation.

By the non-fatal character of the disease, by the absence of bacteremia, by the scantiness of fibrin in the exudate, and by the tendency to earlier resolution, the pneumonia of the non-virulent pneumococcus manifests similarities with the experimental pneumonia produced by insufflation of the streptococcus. However, it differs from the latter by leaving the framework of the lung tissue and the bronchi practically free from injury. While in the advanced lesions caused by the streptococcus there was often a

marked purulent infiltration of the walls of the alveoli and of the bronchioles, of the connective tissue septa, and even of the adventitia of the blood vessels, in the pneumonic lesions brought on by the non-virulent pneumococcus there was at the most only a slight leucocytic infiltration of the walls of the small bronchi.

#### SUMMARY.

The intrabronchial insufflation of a non-virulent pneumococcus causes, like the insufflation of a virulent pneumococcus, the development of an exudate in the lungs which, in general, leaves the framework unaffected, and the lesion presents the gross appearance of a lobar pneumonia. It differs, however, materially from the pneumonia produced by virulent pneumococci in the important points that the consolidation tends to a more rapid resolution, the disease is non-fatal, the blood is not invaded by the organism, and the exudate is strikingly poor in fibrin.

As to the question which was the starting point for the foregoing investigation, namely, whether the pneumonic lesion produced by the streptococcus is merely a form of pneumonia caused by a less virulent organism, it may be answered for the dog, it seems, in the negative. The leucocytic infiltration of the framework of the lungs which occurs invariably in streptococcus pneumonia, and which is practically absent in the lesions caused by the virulent as well as by the non-virulent pneumococcus, is a strong enough feature to form a dividing line between the two forms of experimental pneumonia previously described.

## DER AFFERENTE SPLANCHNIKUS ALS DEPRESSOR.\*

VON JOHN AUER UND S. J. MELTZER.

(Aus dem Department of Physiology and Pharmacology des Rockefeller Institute  
for Medical Research, New York.)

Seit den bekannten Untersuchungen von A s p im L u d w i g s c h e n Laboratorium, jetzt fast ein halbes Jahrhundert, gilt es als eine gesicherte Tatsache, dass Reizung des zentralen Endes des Splanchnikus ausnahmslos eine Steigerung des Blutdruckes bewirkt. Wir wollen hier mitteilen, dass bei neuerlichen Untersuchungen über diesen Gegenstand wir zu genau entgegengesetzten Resultaten gekommen sind: bei Hunden verhielt sich der zentrale Splanchnikus fast wieder Depressor bei Kaninchen. Der Splanchnikus wurde, in den meisten Versuchen, in der Brusthöhle kurz vor seinem Durchtritte durch das Zwerchfell aufgesucht und durchgetrennt und das zentrale Ende mit Induktionsströmen gereizt. Unter Anwendung der intratrachealen Insufflation wurden die Tiere in vollkommener Äthernarkose gehalten. In allen Versuchen waren beide Vagi durchtrennt. In keinem einzigen Falle wurde irgend eine nennenswerte blutdrucksteigernde Wirkung bei der Reizung beobachtet. Dagegen bewirkt die Reizung in der weit aus grössten Mehrzahl der Versuche eine beträchtliche Blutdrucksenkung. In zwei Versuchen, bei denen eine Senkung vermisst wurde, stellte es sich bei der Autopsie heraus, dass nicht alle Zweige durchgeschnitten waren. In einem andern Versuche ohne Senkung — im Anfange der Versuchsreihe — wurde es leider unterlassen, die Autopsie zu machen. Um eine Drucksenkung zu erzielen, mussten die Induktionsreize ziemlich stark sein, etwa 100 bis 75 mm Rollenabstand. Die Senkung war beträchtlich, sie betrug manchmal 60 mm Hg. und darüber, und hielt während einer Reizdauer von 40 bis 50 Sekunden an.

\* Der Redaktion zugegangen am 9. März 1913.



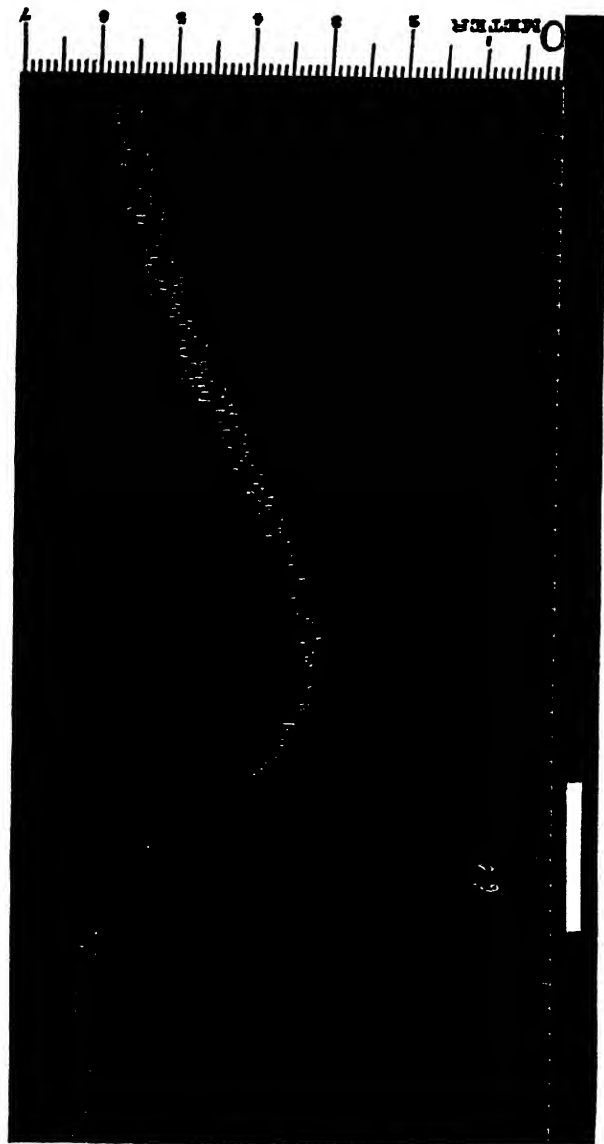


Fig. 1.

27. November 1912. Hund in Äthernarkose, beide Vagi durchgeschnitten, linker Splanchnikus durchgetrennt und das zentrale Ende für 20 Sekunden mit Induktionsströmen gereizt bei 60 mm R. A. Druckabfall von 120 auf 62 Millimeter Quecksilber. Untere Linie: Abszisse und Reizsignal, Zeitmarkierung in 4 Sekunden.

Bei häufiger Wiederholung der Reizungen verminderte sich die Wirkung, um aber nach einer Erholung von 15 bis 20 Minuten auf die ursprüngliche Stärke zurückzukehren. In nur wenigen Versuchen wurde die Reizung nach Durchschneidung der beiderseitigen Splanchnici vorgenommen. Nicht nur fehlte da die Drucksenkung nicht, sie bewirkte sogar in einem Falle einen tödlichen Ausgang. Bis jetzt sind nur wenige Versuche an anderen Tierarten vorgenommen worden. Bei Kaninchen bewirkte eine kurz dauernde Reizung eine Drucksenkung, die aber nur mässig war und bei einer Fortdauer der Reizung in eine Steigerung übergang. Bei Katzen bewirkte die Reizung stets eine Drucksteigerung.

Asp experimentierte an Hunden und Kaninchen. Der Widerspruch zwischen Asps Angaben und unseren Ergebnissen lässt sich vielleicht dadurch erklären, dass Asp seine Versuchstiere, wie er sich ausdrückt, „mit Kurare narkotisiert“ hatte, d. h. sie waren gar nicht narkotisiert, während unsere Versuchstiere vollkommen ätherisiert waren. Nun ist aber der Splanchnikus bekanntlich ein stark sensibler Nerv. Es könnte demnach sein, dass bei Reizung des Nerven bei nicht narkotisierten Tieren die Drucksteigerung, welche die Schmerzen bewirken, stark genug ist, um die Effekte der gleichzeitig gereizten, reinen depressorischen Fasern zu überkompensieren. Wir haben uns aber nicht entschliessen können, diese Vermutung durch das Experiment zu prüfen.

Demnach haben auch die Eingeweide der Bauchhöhle, worin der grössere Teil der Blutmenge sich befindet, ihren Depressor. Es wäre denkbar, dass diese Reflexdilatoren eine bedeutsame regulatorische Rolle spielen. Wenn nämlich durch irgend welche Reizung der afferenten Nervenfasern der Splanchnici eine starke Konstriktion der Gefässe der Baueingeweide bewirkt wird, so könnte die dadurch entstandene Anämie der Eingeweide sich als Reiz auf die afferenten depressorischen Fasern der Splanchnici geltend machen, wodurch Gefässbahnen in anderen Teilen des Körpers für das verdrängte Blut eröffnet werden könnten. Zu dieser Annahme passt die von uns beobachtete Tatsache, dass nämlich Reizung des zentralen Endes

eines Splanchnikus auch dann eine starke Drucksenkung bewirkte, wenn sogar beide Splanchnici durchschnitten waren, die Senkung demnach von Vasodilatationen in anderen Gebieten herrühren müsste, als die, welche vom peripheren Splanchnikus beherrscht werden. Mit anderen Worten: Die von uns aufgedeckten afferenten depressorischen Fasern der Splanchnici stellen vielleicht das „missing link“ dar in dem vielfach angenommenen antagonistischen Mechanismus zwischen dem Blutgehalte der Baueingeweide und dem Volum des Blutes in den übrigen Teilen des Körpers.

## THE REACTION OF THE LUNGS TO THE INTRA-BRONCHIAL INSUFFLATION OF KILLED VIRULENT PNEUMOCOCCI AND OF PLAIN STERILE BOUILLON.\*

By MARTHA WOLLSTEIN, M.D., AND S. J. MELTZER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

In a foregoing communication<sup>1</sup> we reported that the intrabronchial insufflation of a non-virulent strain of pneumococcus produced a lesion which had the gross appearance of a lobar pneumonia. This fact led us to investigate the effects of insufflation of killed virulent pneumococci and of uninoculated sterile bouillon. Both subjects were studied in short series of experiments.

### INSUFFLATION OF KILLED VIRULENT PNEUMOCOCCI.

The problem was investigated in a series of ten dogs. A virulent strain of pneumococcus was grown in broth for about twenty hours, and was then killed by heating to 60° C. for fifteen minutes. Control cultures were made and found to be sterile. Doses of fifteen cubic centimeters of killed pneumococci were introduced into the lungs of six dogs; they showed no signs of illness. Only in one animal was there a rise of temperature of 1° C.

One dog was killed twenty-four hours after inoculation. Its lungs showed no pleurisy, and all the lobes were well aerated except the right posterior, which was dark in its posterior third but neither heavy nor swollen. On section this portion of the lung was very moist; thin, frothy fluid ran from the bronchi, and there was a mottled appearance due to the presence of partly solid areas of small size. By an irregular line of congestion this engorged lung substance shaded into the completely normal portion. Microscopic examination showed the lesion to be one of edema and congestion, with some leucocytic exudate in small groups of alveoli. There was

\* Received for publication, January 9, 1913.

<sup>1</sup> Wollstein, M., and Meltzer, S. J., *Jour. Exper. Med.*, 1913, xvii, 353.

complete absence of fibrin. Cultures from the heart's blood and lung remained sterile. Smears from the lung showed pneumococci in fair numbers. Evidently some of the injected broth containing the dead but still stainable pneumococci was mixed with the frothy fluid exuding from the bronchi. There was no phagocytosis of the pneumococci.

Two dogs were killed forty-eight hours after insufflation. The lungs showed a patchy congestion in the posterior right lobe besides a small, dark, semisolid area (one centimeter square) near the base of this lobe. Microscopically areas of intra-alveolar leucocytic exudation were found, but there was no fibrin in the alveolar contents. Cocci were still demonstrable in smears, but the cultures remained sterile.

In three dogs, which were killed after three days, the lungs were the seat of superficial areas of congestion in the posterior lobes, with no sign of hepatization. Microscopically there was evidence of phagocytosis of red cells and leucocytes by macrophages. The resolving area was a very small one and no hepatized lobules remained.

Four other dogs received doses of dead cocci enriched three times. Their lungs showed similar lesions of congestion, edema, and very small areas of mild inflammation, the lesions being no more extensive nor severe than those resulting from the smaller doses. A very small amount of fibrin was found in the alveolar exudate of a few lobules, polymorphonuclear leucocytes and small numbers of red cells being present as well. Other alveoli contained granular serum alone or with desquamated epithelium. The lesion bore no resemblance to that produced by living, virulent pneumococci in the same length of time.

These experiments then showed that the lesions produced by intra-bronchial insufflation of killed cultures of virulent pneumococci, still containing stainable organisms, are not similar to the massive pneumonias produced by the insufflation of living pneumococci, but consist of inextensive, mild inflammation of a patchy and superficial character. Mild leucocytic exudation into the alveoli, which also contained a few epithelial cells and some serum, was confined to a few lobules. Fibrin was practically absent. The limited exudates were almost completely absorbed on the third day.

## INSUFFLATION OF STERILE BROTH.

In the foregoing experiments the insufflated material contained, besides the broth, substances resulting from the breaking down of some of the pneumococci, and many still morphologically normal, stainable cocci. The question had still to be settled as to the nature of the reaction which the insufflation of sterile broth alone produces. Lamar and Meltzer<sup>2</sup> stated that insufflation of salt solution, broth, serum, etc., was not followed by consolidation. We give briefly some details of the observations made on a small series of animals receiving, by intrabronchial insufflation, uninoculated sterile bouillon of the kind generally used for cultivating pneumococci.<sup>3</sup>

The experiments were made on six dogs. Four of the animals received twenty cubic centimeters of broth each; to the other two fifteen cubic centimeters were given. The dogs did not appear to be ill after the insufflation. The larger dose was followed by a rise of temperature of  $0.7^{\circ}$  C. in two cases, and of  $0.8^{\circ}$  C. in one other instance; but only in the case of one dog did the temperature remain higher than normal during the day following inoculation.

The lungs of the dogs killed twenty-four hours after receiving the broth looked well aerated and pink. In the posterior lobes there were scattered areas of a darker color, giving the lungs a mottled appearance. These darker portions were elastic and aerated. On section they proved to be congested and very moist, exuding a thin, frothy fluid which was chiefly broth; in no case was there any sign of consolidation. The trachea, bronchi, and lymph nodes were normal in appearance. In one instance the posterior half of the right lung was found to be the seat of an extensive hemorrhage, but no inflammation was present. The pleura had retained its lustre perfectly.

Forty-eight hours after the administration of the broth the posterior lobes were still congested, but there was no evidence of consolidation. The dog whose temperature had remained  $0.6^{\circ}$  C. above normal showed very small subpleural hemorrhages over all the lobes of the right lung, and the congestion of the lung substance was intense. There was no edema and no hepatization.

<sup>2</sup>Lamar, R. V., and Meltzer, S. J., *Jour. Exper. Med.*, 1912, xv, 133.

<sup>3</sup>Lamar, R. V., and Meltzer, S. J., *loc. cit.*, p. 136.

On microscopic examination the capillaries in the alveolar walls as well as the larger blood vessels in the lung framework were found to be distended with red blood cells, of which small numbers had escaped into some of the alveoli. Granular material (serum or broth) was found in many alveoli, and there were several groups of air sacs which contained polymorphonuclear leucocytes. The bronchi and the interlobular septa were quite normal.

Cultures made from the lungs and heart's blood of these six animals remained sterile.

These observations show that sterile bouillon causes a pronounced congestion of the lung tissue with which it comes in contact and that exceptionally it may lead to slight hemorrhage. It brings about a negligible amount of inflammation, visible only on microscopic examination. The congestion may last forty-eight hours. It is possible that some of the bouillon remains unabsorbed for that length of time, thus serving as a continuous irritant.

Since all the animals in these two series of experiments had been killed with chloroform, it seemed advisable to examine the lungs of normal, untreated dogs, killed in the same manner. Such an examination showed that the lungs were pink and aerated, uniformly congested in the posterior lobes, and less markedly congested in the middle and anterior lobes. The trachea, bronchi, and lymph nodes were normal. There was no mottling of the lungs with darker patches, and the lungs contained much less blood than did those of the animals treated with bouillon. On microscopic examination the bronchi were all normal; the capillaries were distended with red cells, some of which were found in the alveoli, together with desquamated epithelium. There was no edema, and no leucocytes had left the blood vessels.

The killing with chloroform causes some degree of congestion in the lungs, more marked in both posterior lobes. However, this congestion is definitely less marked than that which follows the injection of broth and which is confined mostly to the posterior lobe of one lung. We may therefore be quite certain that the essential part of the congestion which was found to exist in one of the posterior lobes twenty-four or forty-eight hours after an intrabronchial insufflation of bouillon was due to the irritation of the bouillon present in that part of the lung and not to the chloroform.

#### SUMMARY.

The experiments show that intrabronchial insufflation of a culture of virulent pneumococcus killed by heat and still containing stainable organisms produces an inextensive, mild, patchy, super-

ficial inflammation of the lung tissue bearing no similarity to the lesions produced by the living pneumococcus, and that insufflation of sterile bouillon causes a pronounced congestion of the lung tissue with which it comes in contact, sometimes lasting for forty-eight hours.



## SIMPLE DEVICES FOR EFFECTIVE ARTIFICIAL RESPIRATION IN EMERGENCIES.

By S. J. MELTZER, M.D., LL.D.

*(From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research, New York.)*

In a preliminary note,<sup>1</sup> about a year ago, I described a simple method of artificial respiration which proved efficient in animals. The method consisted in driving air rhythmically by bellows through a tube which has been introduced into the pharynx. The plan of this method was based on the following considerations. Air under pressure in the pharynx has four outlets. It may escape by way of the nasopharynx through the nose. It may escape through the mouth. It may escape through the esophagus into the stomach. It may finally enter into the trachea and the lungs; when it enters there with sufficient force, it causes an inspiration. To obtain an efficient insufflation of air from the pharynx into the lungs, the other exits must be satisfactorily barricaded. In the method described under the name of pharyngeal insufflation the escape of air through the mouth was effectively reduced by pressure on the suprahyoid region. The elastic pharyngeal tube raised the soft palate and the uvula, and thus shut off the entrance into the nasopharynx. The entrance of air through the esophagus into the stomach would, besides the deviation of the air from the lungs, be an additional evil by the gastrectasis and intestinal meteorism which it may cause. All these evils were effectively met in two ways: by introducing a tube into the stomach or by putting a weight on the abdomen. When these various precautions were taken, each insufflation through the pharyngeal tube drove air into the lungs with sufficient force which caused indeed an effective inspiration. The expirations occurred

1. Meltzer, S. J.: Pharyngeal Insufflation, a Simple Method of Artificial Respiration, THE JOURNAL A. M. A., May 11, 1912.

during the interruptions of the insufflation, the elasticity of the chest and of the abdominal viscera driving the air out again into the pharynx from where it escaped through the least resistant passage ways, which were now through the pharyngeal tube and alongside through the unobstructed part of the mouth.

The method thus described, which worked well in four species of animals (dogs, cats, rabbits and monkeys), has since been tried on living as well as on dead human beings. Here, however, the method failed to work. In human beings pressure on the suprahyoid region does not restrict effectively the free escape of air through the mouth; neither is the entrance into the nasopharynx sufficiently blocked by the pressure of the flexible pharyngeal tube. The air insufflated into the pharynx escapes freely through the mouth and nose and enters therefore into the lungs with too little force to overcome the resisting elasticity of the lungs and the thoracic walls and thus to cause an inspiration. In other words the simple arrangement which is so efficient a method of artificial respiration for animals proved to be unsatisfactory when applied to man because of the difference in the anatomic construction. I then set out to develop the method further so as to make it applicable to human beings. I believe that I have now attained that end. In the following I shall describe two methods which have caused effective respiration (that is, effective rhythmic entrance of air into the lungs) even in human cadavers stiff in rigor mortis or frozen stiff. I shall designate the respective methods of artificial respiration as the *pharyngeal* and the *mask device*. I shall describe the pharyngeal device first and in greater detail.

The pharyngeal tube to be used in human beings is made of metal. It measures transversely 38, and vertically 27, millimeters and is about 18 centimeters long. The lower (tongue) side is flat, the upper (palate) side is round. At the pharyngeal end the upper side is longer by about four centimeters than the tongue side; when the tube is inserted through the mouth into the pharynx, the end of the upper side has to reach the posterior wall of the pharynx, while the lower side may end somewhere between the radix of the tongue and the posterior wall of the pharynx. For an adult of medium size the dimensions of the tube are sufficient to fill out the entrance into

the pharynx so as to prevent the escape of air through the mouth; it also blocks reliably the entrance into the nasopharynx. Of course, tubes of various dimensions may be had at hand, so as to fit the individual sizes. The outer end of the tube carries, in the first place, a hollow neck-like projection to connect the tube with the insufflation apparatus. It has, besides, a round hole, through which a large stomach-tube may be introduced into the esophagus and the stomach, when necessary; this hole is usually kept closed by a movable plate.

The outer end of the pharyngeal tube is connected by means of a short heavy piece of rubber tubing with a little device which I designate as respiratory valve. It is a small tube about 10 centimeters long and 3 centimeters in diameter, which carries a valve inside and a ring outside. By means of that ring the valve may be moved from side to side. When it is moved to the right side, it connects the insufflating apparatus with the pharyngeal tube and air or oxygen is driven into the pharynx and the lungs. When the ring is moved to the left side, the current of air or oxygen is shut off; at the same time an opening is established through which the expired air may now readily escape. The respiratory valve may be conveniently held in the hand and the ring moved from side to side by the thumb. The ring moving the valve is not in the middle but near one end of that little device, the end of which should be connected with the pharyngeal tube.

The other end of the respiratory valve should be connected by means of strong rubber tubing with glass-blower foot-bellows which should be worked so as to give an approximately continuous current of air; or it may be connected with an oxygen tank. Between the respiratory valve and the bellows (or oxygen cylinder) a "safety-valve" should be interpolated in order that the air or oxygen should not be driven into the pharynx with too high pressure. The safety-valve may be of such a simple kind as I described in the recently appeared sixth volume of Keen's work on surgery for the intra-tracheal insufflation apparatus. It consists simply of a calibrated tube dipping in mercury. The pressure should be arranged for not less than 20 millimeters mercury, and may be even 25 millimeters; the pharyngeal system of insufflation will always permit an escape of some air through any of the exits.

Heavy weights to be placed on the abdomen, a broad belt to reinforce the pressure on the abdomen, and a large stomach tube, about 33 French, complete the outfit.

The pharyngeal insufflation apparatus for artificial respiration consists then of a metal pharyngeal tube, the respiratory valve, foot-bellows, or an oxygen tank and a safety-valve. In addition there should be on hand a tongue forceps, a stomach-tube, heavy weights and a belt. The procedure is as follows:

After a heavy weight is placed on the abdomen, the tongue should be pulled out by means of an appropriate forceps and the pharyngeal tube inserted into mouth and pharynx as far as it may go. For the



FIG. 1.—Blood-pressure tracing from an etherized dog which received an intravenous injection of sodium nitrite. Blood-pressure low, 44 millimeters; pressure on the abdomen brings up the blood-pressure to 70 millimeters mercury, and the pulse pressure is nearly doubled in size.

sake of being in readiness, the respiratory valve should be kept attached to the pharyngeal tube. The free end of the respiratory valve should now be connected with the foot-bellows or the oxygen tank to either of which a safety-valve is attached. Now the oxygen tank should be opened, or the foot-bellows started working, while the respiratory valve is taken in the right hand and the ring moved by the thumb from side to side, keeping it for two or three seconds at each place. The same man who works the bellows with his foot may work at the same time the respiratory valve with his hand.

When the ring rests at the right side, the air from the bellows or the oxygen from the tank is insufflated into the pharynx with a force of 20 millimeters of mercury and unavoidably enters the lungs, causing an inspiration. On account of the pressure on the abdomen the inspiration causes essentially distention of the thorax. When the ring is turned to the left the insufflation is cut off and the elastic recoil of the ribs and of the abdominal viscera causes an efficient expiration. The rubber tubing connecting the pharyngeal tube with the respiratory valve should be short, in order to cut off the dead space and during expiration eliminate carbon dioxide as much as possible. Rebreathing is surely undesirable. All tubing employed for connections should have thick walls to prevent kinking. The tongue should be kept pulled out in order to keep the epiglottis raised. After the pharyngeal tube is inserted the tongue may be kept in proper position by tying it (not too tight) to the tube; the forceps may be then taken off. The tying of the stretched tongue to the tube may even assist the latter in remaining in position.

The weight on the abdomen prevents the entrance of air in any considerable quantity into the stomach and the little which gets there escapes again when the insufflation is cut off; it never gets into the intestines. The pressure on the abdomen has still another significance. In patients with completely abolished respiration usually the blood-pressure is also very low and most of the blood may be accumulated in the abdominal viscera. The heart is then scantily filled, and not enough arterial blood is sent to peripheral organs. Under such circumstances a good pressure on the abdomen may raise the blood-pressure by even as much as 30 millimeters of mercury; the heart is filled more efficiently and sends more blood to the medulla oblongata, arousing there the activities of the respiratory and vasomotor centers. Figure 1 shows the effect of abdominal pressure on the blood-pressure.

For this reason I recommend to have a belt on hand to reinforce the pressure. With a belt alone not much success can be obtained. In cases of accidents, when it might happen that no suitable weight is at hand, the individual who handles the respiratory valve may sit down on the abdomen of the victim.

There might be conditions which do not permit the placing of

weights on the abdomen; for instance, when a collapse occurs during a laparotomy. Under this circumstance a stomach-tube of a large diameter should be introduced through the esophagus into the stomach. The tube restricts to a sufficient degree the entrance of air into the stomach, and the air which enters there escapes readily through the tube. As stated before, in the anterior end of the pharyngeal tube is an opening for that purpose which is usually kept closed by a movable plate. A stomach-tube of a 33 French diameter fits exactly into this opening. I am of the opinion that it is preferable to have in every instance, even when pressure on the abdomen is exerted, also a tube in the stomach. Since the apparatus may have to be used in some emergency cases by laymen, however, the latter might be loath to handle a stomach-tube. And since the experiments have shown that very good results may be had with the pressure alone I do not feel like insisting on the simultaneous use of the stomach-tube in all simple cases.

Besides the metal pharyngeal tube, I studied also the availability of the use of insufflation with the aid of a well-fitting mask. In this arrangement every other part is the same as in that for the pharyngeal tube, except that instead of introducing a tube into the pharynx, a mask is laid over mouth and nose and by bands tightly applied to the face. The mask has a hollow projection for the connection with the respiratory valve. I tested the mask method on various animals; as was previously found for the pharyngeal tube, it was established that also by means of the mask efficient artificial respiration can be carried on. With the aid of the mask method of artificial respiration, completely curarized (and anesthetized) animals were kept in an excellent condition for many hours. By this method, of course, some infectious matter may be driven into the trachea and perhaps cause infection; by this method, further, pressure is exerted on the middle ear; neither does the mask method allow the introduction of a stomach-tube. However, in dealing with emergency cases, with immediate danger to life, such considerations as the above mentioned are, comparatively speaking, mere trifles and can hardly be taken into account.

I have tested also the effectiveness of insufflation through metal pharyngeal tubes on animals; it is even more satisfactory than with

elastic rubber tubes. It works promptly; the introduction gives less trouble and the tube remains in position for hours.

Both the pharyngeal and mask methods were tested also on human cadavers. Air entered into the lungs when insufflated by either of these methods, even if the dead bodies were in rigor or frozen stiff. In some cases unmistakable efficient respiratory movements of chest and abdomen were manifestly present. But even when the stiffness interfered with the free movements, auscultation proved conclusively the entrance of air into the lungs. Especially was this the case in a man who died under signs of pulmonary edema; râles could be heard all over the chest.

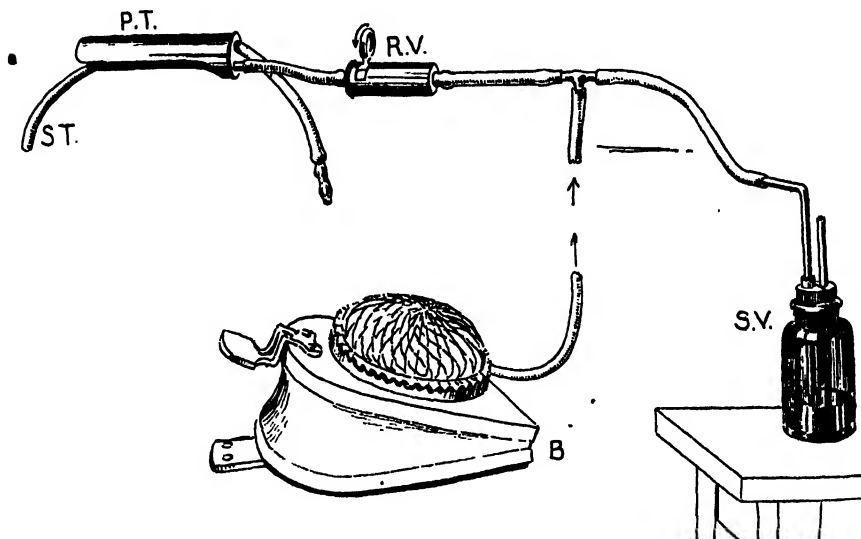


FIG. 2.—Arrangement of the pharyngeal device for artificial respiration. P. T., pharyngeal tube. R. V., respiratory valve. The ring turns the valve; turning to the right (facing the pharyngeal tube) brings an inspiration and to the left brings an expiration. B, foot-bellows. S. V., safety-valve. The bottle of the safety-valve should be shorter and have a wider diameter than the one in the figure; it is less likely to turn over. S. T., stomach-tube introduced through the opening in the pharyngeal tube.

The accompanying sketches illustrate these methods better than they can be described. In Figure 2, the pharyngeal tube (P. T.) is shown connected with the respiratory valve (R. V.), the foot-bellows (B) and the safety-valve (S. V.). A stomach-tube (S. T.)

is pushed through the pharyngeal tube. In Figure 3 the mask (M.) is shown applied to the face. By means of an inflatable ring (Infl.) the mask is made air tight. There is a weight on the abdomen and a belt around it. The respiratory and safety valves are the same as in Figure 2. The bellows are here replaced by an oxygen cylinder.

In an emergency case no time should be lost on matters of less importance before starting the main act, and that is: The artificial respiration. When using the mask, for instance, no time should be lost in tying and fixing it properly; it should be pressed over

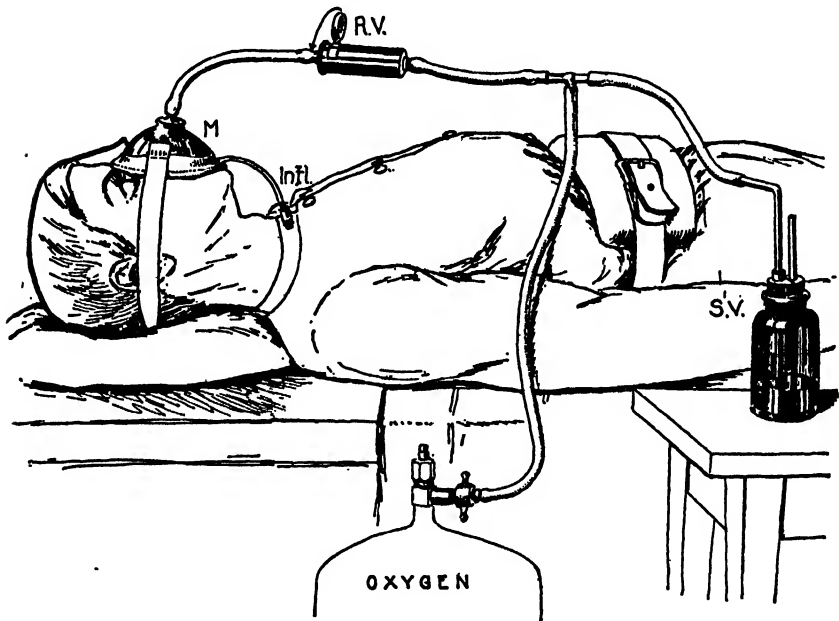


FIG. 3.—M., mask. Infl., tube for inflating the rubber ring around the rim of the mask. R. V., respiratory valve. S. V., safety-valve. An oxygen cylinder provides here the insufflation pressure. The figure shows also the weight on the abdomen and the belt around it.

mouth and face by the hand. After the insufflation is well on the way, some one may attend to the tying of the mask, the fixing of the tongue properly and the putting of the belt around the weight over the stomach. Regarding the fixing of the tongue, it may be,



as stated before, tied to the pharyngeal tube, when using the same. When using the mask, the handle of the tongue forceps may be fixed to the victim's neck so as to keep the tongue stretched; or the tongue may be tied by means of tape or gauze bandage, pulled out well and the end of the tape or bandage tied around the victim's neck. It should be kept in mind that the pulling out of the tongue is an essential factor in any procedure for artificial respiration. In completely paralyzed individuals there is a tendency for the tongue to be kept somewhat firmly over the entrance into the larynx, caused, perhaps, by some final attempt at inspiration. I may say in passing that the demonstrations made with some machines for artificial respiration, for commercial and advertising purposes, on living and unanesthetized individuals, are entirely misleading and should not be taken as evidence of the efficiency of such machines in cases when individuals are unconscious and the respiratory mechanism paralyzed.

It will be safer to have on hand a mask as well as a pharyngeal tube. When the latter should prove too small, the escape of the insufflated air alongside the tube may be remedied by tamponading the entrance into the pharynx around the tube with gauze. Besides, as I have indicated above, tubes of various sizes should be on hand.

The foot-bellows need not be large; smaller foot-bellows worked a little more rapidly give a sufficiently strong continuous current of air. The continuous air current is in the arrangement here described preferable to the interrupted current produced by hand-bellows; it is difficult to have the rhythm of the bellows coincide properly with the rhythm in the respiration produced by the respiratory valve; it may occur that the bellows are compressed just when the valve is closed, etc., and the result might be an irregular and inefficient artificial respiration.

It is evident that the methods of artificial respiration by devices here described can be readily combined with the Schäfer method of manual artificial respiration. The individual is then placed on his abdomen and the turning of the ring of the respiratory valve to the left has to coincide in time with the pressure on the lumbar muscles. Inspiration as well as expiration will thus be efficiently reinforced.

While on the basis of my extensive experience I have reason to believe that the devices which are here described will answer the purpose satisfactorily in all cases in need of artificial respiration, it is safer to think of methods which are capable of improving the efficiency of the devices. Emergencies may arise of which we are unable to think now; factors of safety are designated by some students of mechanics as factors of ignorance.

The emergency bag should contain small foot-bellows, the safety-valve, the respiratory valve and the pharyngeal tube all readily connected by rubber tubing. Further, a mask, a tongue forceps, a strong belt or cords, a stomach-tube, a roll of tape or one-inch gauze bandages and scissors. Weights might increase too much the weight of the bag. Bricks, stones or pieces of heavy metal, etc., may be had at any place. At any rate, a strong one-inch thick wooden board, 8 by 6 inches, placed on the abdomen and pressed down well by means of two belts (or bandages), one at each end, will do the same service as heavy weights and will be a good deal lighter to carry. Short pieces of glass tubes, T-tubes and pieces of rubber tubing should be carried in reserve. Such a bag need not weigh more than ten pounds.

Wherever oxygen can be had it should be used in preference to the air from the bellows. It should be remembered that according to Hill and Macleod, however, prolonged inhalation of oxygen may do harm to the lungs. When, therefore, prolonged artificial respiration is required, the use of air should be alternated with oxygen.

The devices for artificial respiration here described are certainly simple and inexpensive. Their efficiency has been tested to a much greater extent than any other device I know of. The possibility of keeping up the circulation in a normal condition for hours while the voluntary respiration is completely abolished (by curare) is certainly a rigid test, which has not been applied to any other method of artificial respiration except to that used in experimental laboratories with tracheotomy as a prerequisite, and, as I may add, to the method of intratracheal insufflation. The last mentioned device, which has now been tested in nearly two thousand cases on human beings, would be, in my opinion, indeed the most ideal method

for artificial respiration. It has been used, to my knowledge, in two human cases of severe poisoning (morphin .15 grains subcutaneously combined with inhalation of gas, and smoking opium for two days with complete absence of respiration) for twelve hours continuously with complete recoveries. But this method requires some training and could never be left to the hands of laymen. The handling of the artificial respiration by means of the pharyngeal and mask devices which I describe here is so simple that laymen could well be entrusted with its execution. And that was the main object of my endeavor to develop these devices. Cases of collapse from injuries, of poisoning by gas, of shock by electricity, etc., are discovered most frequently at places where there is no efficient medical help at hand, and time is here very precious. With the minutes which elapse without adequate help the chances for recovery diminish rapidly. These chances will increase when the devices for resuscitation are inexpensive, so that they could be had and be in readiness in larger numbers in many places, and are so simple that the execution of the resuscitation by means of these devices could be carried out by many laymen with success.

The necessity of having reliable devices for artificial respiration, however, is not confined to mines, to mills of all kinds, or to electric plants. They are surely of great necessity in hospitals. Not infrequently a surgical patient could be saved if artificial respiration could be carried on efficiently for some time. There are also numerous medical cases in which simple and efficient artificial respiration would prove life-saving. Temporary respiratory insufficiency or paralysis from any source (poliomyelitis, postdiphtheritic paralysis, tabes, eclampsias of all kinds, etc.), are not rare phenomena. And why not apply the mask method with oxygen and abdominal compression in many acute cases with inefficient circulation and respiration? "There is the possibility that the actual cause of death might be, in one case or another, especially in acute cases, only of a temporary nature, so that efficient artificial respiration might assist in temporizing and thus prove occasionally life-saving indeed. Such possibilities, though they may be realized only once in a thousand times, justify the making of an attempt in each and every instance."

Life depends essentially on the efficiency of the functions of respiration and circulation. All deaths are due in the last analysis to the failure of either of those two functions. Reliable devices by means of which the function of respiration can be kept up efficiently for many hours are bound to be the means of saving many lives. I am confident that the method of intratracheal insufflation as well as the pharyngeal and the mask methods described here are such reliable devices for efficient artificial respiration.

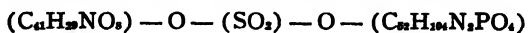
## THE SULPHATIDE OF THE BRAIN.\*

BY P. A. LEVENE.

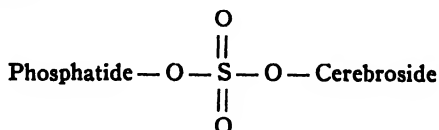
(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

There exists very little definite information regarding the nature of the sulphur-containing lipoids. The authors who are defending the individuality of protagon assume that both sulphuric and phosphoric acids enter into the structure of its molecule. On the other hand, Thudichum,<sup>1</sup> on the ground of theoretical considerations, was led to the belief that the sulphatide is a distinct lipoid having some properties in common with the phosphatides. Unfortunately he failed to separate the two substances. The ratio of sulphur to phosphorus in the purest sample analyzed by this author was 3:2, in other samples the ratio was 1:1.

In very recent years W. Koch<sup>2</sup> made the sulphatide the subject of a new investigation. Koch arrived at the conclusion that the sulphatide contained an equimolecular proportion of phosphoric and of sulphuric acids. The analytical data led him to formulate the structure of the substance in the following manner:



or, more graphically,



Thus the "phosphatide-cerebroside-sulphatide" of Koch contained all the elements that were supposed to be parts of protagon, and in a way the substance may have been regarded with some

\* Received for publication, November 21, 1912.

<sup>1</sup> *A Treatise on the Chemical Constitution of the Brain*, London, 1884.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, lxx, p. 94, 1910.

degree of justice as the purest protagon, although W. Koch militated against the existence of this complex.

In course of an investigation into the lipoids of the brain we found it possible to isolate the sulphur-containing lipid of the brain entirely free from phosphatides. In its physical appearance and in many of its properties the substance possessed a great resemblance to other lipoids and had the following composition:

0.1216 gram of the substance gave 0.2714 gram  $\text{CO}_2$  and 0.1160 gram  $\text{H}_2\text{O}$ .  
 0.1216 gram of the substance gave 0.2706 gram  $\text{CO}_2$  and 0.1150 gram  $\text{H}_2\text{O}$ .  
 0.2000 gram of the substance employed for Kjeldahl nitrogen estimation required for neutralization 3.3 cc. of  $\frac{N}{10}$  sulphuric acid.

0.4000 gram of the substance used for a sulphur estimation gave 0.0766 gram of barium sulphate.

0.4000 gram of the substance used for phosphorus estimation gave a negative result.

A comparison of the results of the analysis of the samples here described, and of those obtained by Thudichum and by Koch shows the following results:

	THUDICHUM	KOCH	LEVENE
C.....	47.70		60.90
H.....	7.54		10.67
N.....	1.14		2.31
P.....	3.94	1.80	0.00
S.....	6.19	1.91	2.66
O.....	33.49		23.46

The substance is dextrorotatory and melts at  $210^\circ \text{C}$ . (uncor.).

By the method employed for the preparation and purification of the sulphatide it was not possible to further purify the substance. Other methods will have to be devised for that purpose.

It must be remarked here that both Thudichum and Koch found that human brain offered a more suitable material for the preparation of their sulphatide. The substance described in this communication was isolated from beef brains.

Further work on the chemical structure of the substance is in progress.

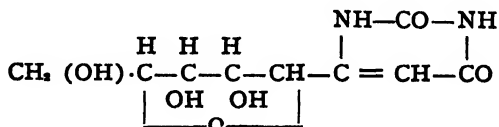
## ON NUCLEASES.

### THIRD PAPER.\*

BY P. A. LEVENE AND F. B. LA FORGE.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

It was demonstrated through the work of Levene and Jacobs<sup>1</sup> that, compared with purine ribosides, the pyrimidine ribosides possessed a much higher resistance towards the hydrolytic action of mineral acids, and on the basis of this property a method was devised for the isolation of pyrimidine bodies. It was later shown by Levene and Medigreceanu<sup>2</sup> that a similar difference existed between the two classes of ribosides in regard to their behavior towards certain enzymes. Thus it was found that nucleosidases, which hydrolyzed the purine derivatives, remained without action on the pyrimidine bodies. In a previous work by<sup>3</sup> the present writers the fact was brought to light that the difference in the behavior of the two classes of substances towards dilute mineral acids can be removed by the reduction of the pyrimidine base in the pentoside to the corresponding dihydropyrimidine. Thus, while uridine was resistant to boiling with dilute aqueous mineral acids, dihydro-uridine behaved under these conditions similarly to inosine, or adenosine, or to any other glucoside.



Uridine,

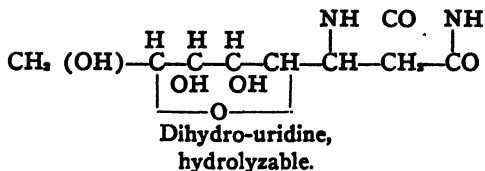
non-hydrolyzable by dilute acids.

\* Received for publication, December 4, 1912.

<sup>1</sup> Levene and Jacobs: *Ber. d. deutsch. chem. Gesellschaft.*, xliii, p. 3150, 1910.

<sup>2</sup> Levene and Medigreceanu: this *Journal*, ix, p. 65, 1911; ix, p. 389, 1911.

<sup>3</sup> Levene and La Forge: *Ber. d. deutsch. chem. Gesellschaft.*, xlv, p. 608, 1912.



Thus, dihydro-uridine was shown to possess a chemical structure in every way analogous to other nucleosides, and hence presented a convenient medium for the study of the specificity of nucleosidases. Thus, if it were possible to find tissue extracts which would hydrolyze the nucleosides of one group and failed to decompose the substances of the other group, this find would contain the proof of the existence of specific enzymes capable of acting on nucleosides of only one definite group. In reality it was found that the tissue that possessed the most pronounced action on the purine nucleosides remained without action on dihydro-uridine, and as yet it is impossible to find an enzyme capable of hydrolyzing either the original uridine, or dihydro-uridine. Hence, the mechanism by which the organism brings about the dissolution of pyrimidine nucleosides is as yet undiscovered, but it is made certain that individual nucleosidases possess a limited specific activity.

#### EXPERIMENTAL PART.

In the experiments here recorded the extract of the intestinal mucosa was used as an enzyme solution. It was prepared in the manner described in the papers by Levene and Medigreceanu.

The activity of the extract was tested on adenosine solution and on dihydro-uridine. The presence or absence of hydrolysis was ascertained by the changes in the optical activity of the solution in different intervals after the beginning of the experiment and by the power of the reaction product to reduce Fehling's solution.

The solutions of enzyme and nucleoside were made up to contain one per cent of Henderson's phosphate mixture.

Toluene was used as antiseptic in all experiments.

*Experiment 1.*—(a) About 0.4 gram of dihydro-uridine was dissolved in 7.0 cc. of water, 1.0 cc. of a 10 per cent Henderson's phosphate mixture and 2.0 cc. of the enzyme solution were added.



Original rotation,  $[\alpha]_D = -0.50^\circ$   
 After 24 hours,  $[\alpha]_D = -0.50^\circ$

(b) 4.0 cc. of a cold saturated aqueous solution of adenosine were diluted with 0.5 cc. of a 10 per cent phosphate mixture and 0.5 cc. of the enzyme solution.

Initial rotation,  $[\alpha]_D = -0.45^\circ$   
 After 24 hours,  $[\alpha]_D = -0.20^\circ$

(c) 1.0 cc. of Henderson's phosphate solution + 2 cc. of enzyme solution and 7 cc. of water.

Initial rotation,  $[\alpha]_D = +0.05^\circ$   
 After 24 hours,  $[\alpha]_D = +0.05^\circ$

The dihydro-uridine solution gave very slight reduction on prolonged boiling with Fehling's solution. An increase in the reducing power at the end of the experiment could not be noticed. The adenosine solution showed no reduction at the beginning of the experiment and a marked reduction at the end.

The enzyme solution had no reducing power.

*Experiment 2.*—(a) To 5 cc. of 25 per cent solution of dihydro-uridine was added 0.5 cc. of the enzyme solution.

Initial rotation,  $\alpha_D = -3.82 (\pm 0.02)$   
 After 48 hours,  $\alpha_D = -3.88 (\pm 0.02)$

(b) Enzyme solution did not possess any appreciable optical activity.

(c) About 1.0 gram of adenosine was suspended in 20.0 cc. of 1 per cent phosphate mixture and 2.0 cc. of enzyme solution; since a part remained insoluble it was not possible to measure the optical rotation of the substance. The action of the enzyme solution on adenosine was ascertained by the reducing power of the solution on Fehling's solution.

The dihydro-uridine showed no change in reducing power before and after enzyme action.

Adenosine solution possessed no reducing power on Fehling's solution at the beginning of the experiment and a very marked reduction after forty-eight hours.

The enzyme solution showed no reducing power either at the beginning or at the end of the experiment.

## ON THE ACTION OF LEUCOCYTES ON SOME HEXOSES AND PENTOSEs.

### THIRD COMMUNICATION. CONTRIBUTION TO THE MECHANISM OF LACTIC ACID FORMATION FROM CARBOHYDRATES.\*

BY P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

In previous communications the authors have demonstrated that, through the action of leucocytes, glucose is transformed into *d*-lactic acid, and that the cleavage of the sugar molecule does not proceed beyond this phase. Since then, the results obtained by us were corroborated by Embden and his co-workers, Kondo and K. v. Noorden, Jr.,<sup>1</sup> and by Rona and Arnheim.<sup>2</sup>

Regarding the conditions of the experiments it was stated that a certain degree of hydroxyl concentration, such as is offered by a 1 per cent Henderson phosphate mixture, was required for a successful result, and that distilled water could not be used as a medium of the reaction, since it prevented the reaction from taking place, and antiseptics, such as toluene and chloroform, acted in the same way. At the time of our first publication we had overlooked a statement of Rona and Döblin<sup>3</sup> that the glycolysis generally produced by blood was absent when blood had been diluted with distilled water or when chloroform had been added to it.

The observations on the action of leucocytes were extended to a larger number of sugars, both hexoses and pentoses, with a view of elucidating the mechanism by which, in the organism, lactic acid is formed from sugar. It is obvious that a molecule of hexose cannot

\* Received for publication, February, 3, 1913.

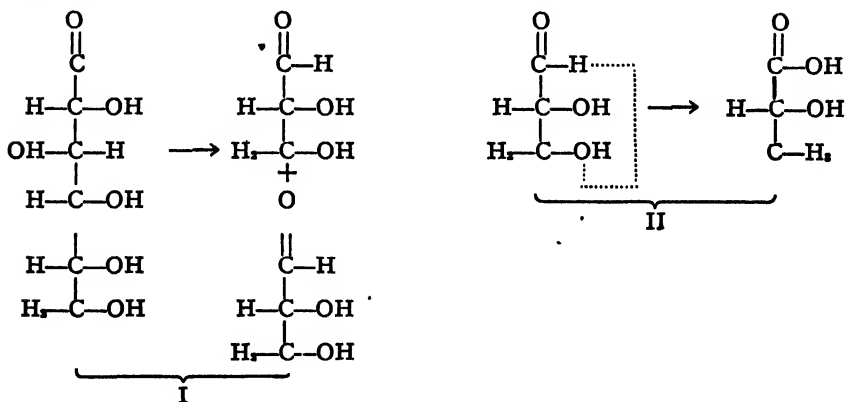
<sup>1</sup> *Biochem. Zeitschr.*, xlv, pp. 63 and 94, 1912.

<sup>2</sup> *Ibid.*, xlviii, p. 35, 1913.

<sup>3</sup> *Ibid.*, xxxii, pp. 489-508, 1911.

undergo a direct decomposition into two molecules of lactic acid without passing a number of intermediary transformations. In recent years there have been brought to the front a great many speculations tending to elucidate the mechanism of the reaction. They can be found in recent publications on alcoholic fermentation, lactic acid fermentation or on glycolysis.

After the publication of our previous articles on the action of leucocytes on glucose, and while the present investigation was in progress, there appeared a series of articles by Embden and his co-workers, on the basis of which Embden formulated a theory that by the action of tissue enzymes one molecule of glucose is dissociated into two molecules of glyceric aldehyde, second, that glyceric aldehyde is then transformed into lactic acid, and third, that the  $\alpha$ -carbon atom of the glyceric aldehyde remains unaffected in course of the transformation. The mechanism may be presented in the following form.

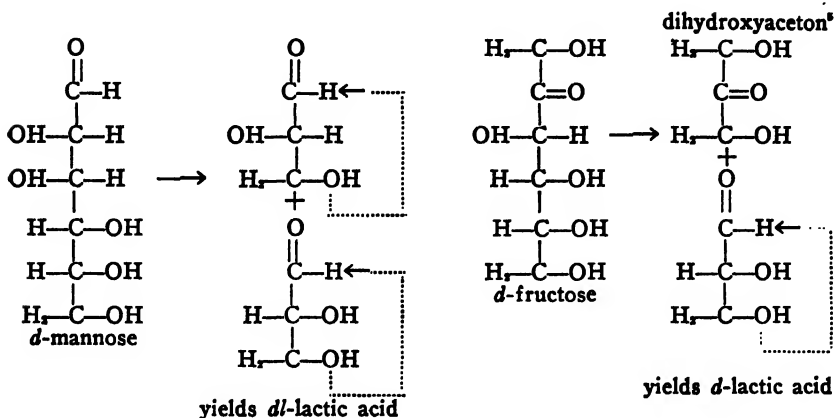


Although our own work on the mechanism of the reaction is not yet completed, yet it seems to us, that it furnishes a very definite argument against the correctness of Embden's view.<sup>4</sup>

It is obvious that if a molecule of glucose dissociates into two of lactic acid, according to the hypothesis of Embden, both of the latter possess the same optical character. Thus, *d*-glucose may yield two molecules of *d*-lactic acid. In this case, however, mannose and fructose would not be expected to yield the same result. It is evi-

<sup>4</sup> *Biochem. Zeitschr.*, xlv, p. 108, 1912.

dent from a glance at the configuration of the respective sugars that mannose would be expected to form *dl*-lactic acid, and fructose a mixture of *dl*- and *d*-lactic acid.



It was found in the course of our experiments that fructose, mannose and galactose all are transformed into lactic acid by means of leucocytes under the conditions reported in the previous communications. Regardless of the nature of the hexose, the lactic acid formed was invariably the *d*-form. Of course, it is important to make certain that the lactic acid obtained in the experiment did not consist of a mixture of the active and inactive forms. This possibility can be excluded in our experiments on the following grounds: first, the zinc salt of the *dl*-lactic acid is more insoluble than that of the optically active form; hence, it should be the first to crystallize if it were present in the mixture; second, the specific rotation of the zinc salt obtained from any one of the hexoses was of the same magnitude. Thus, on the basis of these experiments it does not seem possible that the formation of lactic acid is brought about by simple rearrangement in the molecule of glyceric aldehyde. Whether or not pyruvic aldehyde is the phase immediately preceding the formation of lactic acid remains to be established.

Attempts to bring about a dissociation of pentoses by means of leucocytes resulted negatively.

It may be mentioned here that tissues preserved under strict aseptic

\* Would be expected to yield *dl*-lactic acid.

conditions act on sugars in a way identical to leucocytes. The results of the experiments in that connection will be communicated separately.

#### EXPERIMENTAL.

*Leucocytes.*—These were obtained from dogs by injections of turpentine into the pleural cavity. The technique and further handling of the material has been described in a previous communication.

*Solutions.*—The suspensions were made in the identical manner as the glucose experiments, in a 1 per cent Henderson phosphate solution.

*Bacteriological Controls.*—Both aerobic and anaerobic cultures were made from all leucocyte-sugar mixtures at the close of the experiment and only those analyzed which were sterile. The bacteriological examinations were made by Dr. J. Bronfenbrenner and we take this occasion to express our appreciation.

*Methods of Analysis.*—Sugar was estimated by reduction of Fehling's solution; the reduced copper was determined by Volhard's method.

*Lactic Acid.*—Since the previously reported experiments we have succeeded in obtaining a von der Heide ether extraction apparatus.<sup>6</sup> The leucocyte sugar solutions were carefully made neutral to litmus, brought to a boil and the proteins coagulated with the addition of very dilute phosphoric acid. The addition of sodium sulphate crystals facilitates the precipitation of the proteins. The solution was filtered and again made neutral to litmus, and then sufficiently concentrated *in vacuo* to be contained in the ether extractor. Crystalline sodium sulphate is added to give about a one-half saturated solution and then 5 to 10 c.c. of phosphoric acid are added. Extraction is allowed to proceed for at least seventy-two hours. The ether extract is then dried over anhydrous sodium sulphate and filtered. Water is added to the flask and the ether distilled off. The lactic acid in aqueous solution is converted into the zinc salt in the usual manner.

<sup>6</sup> *Bericht d. königl. Lehranstalt f. Weinbau in Geisenheim a. Rh., 1906, p. 23.*

# I. Experiments Showing Disappearance of Sugars in Mixture of Sugar and Leucocytes.

## Mannose.

	SOLUTION USED	NH <sub>4</sub> CNS	NH <sub>4</sub> CNS PER CC.	SUGAR	LOSS	PER CENT LOSS
	cc.	cc.		per cent		
a. At beginning of experiment.....	1	20.6	20.6	6.35		
After thirty-six hours.....	1	19.8	19.8	6.10	0.25	4.10
b. At beginning of experiment.....	2	36.4	18.2	5.62		
After thirty-six hours.....	2	34.8	17.4	5.36	0.26	4.86
c. At beginning of experiment.....	1	13.6	13.6	4.19		
After thirty-six hours.....	1	12.0	12.0	3.69	0.50	11.90
d. At beginning of experiment.....	1	20.0	20.0	6.16		
After thirty-six hours.....	1	17.6	17.6	5.42	0.74	12.00
e. At beginning of experiment.....	1	19.0	19.0	5.85		
After thirty-six hours.....	1	16.3	16.3	5.03	0.82	14.00

## Laevulose.

a. At beginning of experiment.....	2	37.2	18.6	6.40		
After thirty-six hours.....	2	35.4	17.7	6.09	0.31	5.10
b. At beginning of experiment.....	2	35.0	17.5	6.02		
After thirty-six hours.....	2	33.4	16.2	5.56	0.46	7.35
c. At beginning of experiment.....	1	17.6	17.6	6.05		
After thirty-six hours.....	1	16.4	16.4	5.48	0.53	8.40
d. At beginning of experiment.....	1	15.9	15.9	5.41		
After thirty-six hours.....	1	14.5	14.5	4.90	0.51	8.95

## Galactose.

a. At beginning of experiment.....	2	30.8	15.4	5.61		
After thirty-six hours.....	2	29.8	14.9	5.43	0.18	3.20
b. At beginning of experiment.....	2	28.0	14.0	5.09		
After thirty-six hours.....	2	26.2	13.1	4.77	0.32	6.29

## Arabinose.

a. At beginning of experiment.....	2	29.2	14.6	5.69		
After thirty-six hours.....	2	29.2	14.6	5.69	0	0
b. At beginning of experiment.....	2	28.2	14.1	5.50		
After thirty-six hours.....	2	28.4	13.2	5.53	0	0

## Xylose.

a. At beginning of experiment.....	2	27.6	13.8	5.38		
After thirty-six hours.....	2	27.6	13.8	5.38	0	0

# II. Experiments Showing Formation of d-Lactic Acid during "Glycolysis" of Mannose.

a. 150 cc. of the leucocyte mannose mixture (c, Experiment I) were extracted in a von der Heide extractor with ether. Yield of crude lactic acid = 0.2134 gram.

0.145 gram of the recrystallized salt in 2.167 grams of water in a 1 dm. tube gave a rotation,  $\alpha = -0.45^\circ$ .

$$[\alpha]_D^{20} = -6.7^\circ$$

b. 300 cc. of leucocyte mannose mixture (*d* and *e*, Experiment I) were together extracted with ether in a von der Heide extractor.

0.563 gram anhydrous zinc lactate was obtained. This was recrystallized and analyzed.

0.2278 gram of the recrystallized salt lost, on drying to constant weight at  $110^\circ$ ,

0.029 gram  $H_2O$  = 12.73 per cent  $H_2O$ .

Calculated for two molecules  $H_2O$  = 12.88 per cent.

0.0906 gram anhydrous salt after ignition

gave 0.0302 gram  $ZnO$  = 33.33 per cent  $ZnO$ .

Calculated = 33.40 per cent.

0.1344 gram zinc salt in 1.8684 grams  $H_2O$  gave a rotation in a 1 dm. tube of  $\alpha = -0.47^\circ$ .

$$[\alpha]_D^{20} = -7.0^\circ$$

c. 300 cc. of laevulose leucocyte mixture (*c* and *d*) were extracted with ether in a von der Heide extractor. The yield of recrystallized zinc lactate = 0.2474 gram.

0.1624 gram recrystallized salt lost, on drying to constant weight, 0.0211 gram

$H_2O$  = 12.95 per cent  $H_2O$

Calculated for two molecules  $H_2O$  = 12.88 per cent.

0.1413 gram anhydrous salt was ignited and

gave 0.0469 gram  $ZnO$  = 33.20 per cent  $ZnO$ .

Calculated = 33.40 per cent.

0.1536 gram zinc salt in 2.0138 grams  $H_2O$  gave a rotation in a 1 dm. tube of  $\alpha = -0.49^\circ$ .

$$[\alpha]_D^{20} = -6.8^\circ$$

## ON CEREBRONIC ACID.

### SECOND PAPER.\*

By P. A. LEVENE AND C. J. WEST.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

On the basis of experiments published in a previous communication Levene and Jacobs<sup>1</sup> reached the view that cerebronic acid had the structure of an  $\alpha$ -hydroxypentacosanic acid. On reduction the acid formed a hydrocarbon melting at  $54^{\circ}$ – $57^{\circ}$  C. The melting point for the normal pentacosan, according to Krafft and Marie, should be  $53.5^{\circ}$ – $54^{\circ}$ . Hence it remained uncertain whether the discrepancy in the melting points was due to the fact that the hydrocarbon obtained from cerebronic acid contained some impurity or to a structural difference in the normal pentacosan and in the hydrocarbon obtained from cerebronic acid. It was, therefore, concluded to prepare a larger quantity of the material, which would permit of a more perfect purification of the hydrocarbon.

This was accomplished with the result that on reduction of cerebronic acid a hydrocarbon was obtained that melted at  $53^{\circ}$ – $54^{\circ}$  C., which harmonizes with the melting point corresponding to the normal pentacosan.

The normal nature of the carbon chain of cerebronic acid was corroborated further by the fact that the acid,  $C_{24}H_{48}O_2$ , obtained on the oxidation of the former was reduced to a hydrocarbon melting at  $51^{\circ}$ – $52^{\circ}$  C., and that Krafft found  $51.1^{\circ}$  C. to be the melting point of the normal tetracosan. Hence, it may be considered proven that cerebronic acid is a normal  $\alpha$ -hydroxypentacosanic acid.

In course of the present investigation great care was taken in establishing the exact condition for preparation of the most impor-

\* Received for publication, February 19, 1913.

<sup>1</sup> This *Journal*, xii, p. 381, 1912.



tant derivatives of the acid, also of the methods of separation and of purification of the acid.

#### EXPERIMENTAL PART.

##### *Preparation of Cerebronic Acid.*

The mixture of cerebronic acid and ester obtained from the hydrolysis of cerebrine<sup>2</sup> is heated with an excess of alcoholic sodium hydroxide for four hours, during which most of the sodium salt separates out. The mixture is cooled, the soaps filtered off and washed with methyl alcohol and ether. The sodium salt is then recrystallized from boiling ethyl or methyl alcohol. The cerebronic acid is liberated by suspending the salt in dilute hydrochloric acid and heating on the water bath until the free acid has completely melted. When cold this is filtered off, recrystallized from alcohol and the excess of alcohol removed by melting in vacuum on the water bath. The product thus obtained is pure enough for most operations. It will not, however, give sharp values when titrated to determine the molecular weight. The acid used for this purpose must be further purified through the lead salt. The hot methyl alcoholic solution of the acid is treated with a hot solution of lead acetate in the same solvent as long as a precipitate forms. Finally a few drops of concentrated ammonia are added to neutralize the free acetic acid. After being cooled the lead salt is filtered off and washed with a little warm methyl alcohol. It is then suspended in warm, dry toluene and decomposed by treating with hydrogen sulphide for two hours. The mixture should be kept warm on a water bath and constantly stirred during this time. The lead sulphide is allowed to settle, the toluene solution of the acid filtered off, the sulphide washed with warm toluene and the filtrate concentrated in vacuum. The colorless residue is then recrystallized from absolute alcohol and the excess of alcohol removed on the steam bath. If ammonia is not added in the precipitation of the lead salt the sulphide may come down in a colloidal form. In this case, the entire mixture is concentrated in vacuum and the acid extracted from the residue with a large quantity of boiling alcohol.

<sup>2</sup> Levene and Jacobs: *loc. cit.*, p. 383.

The acid thus prepared melted at  $77^{\circ}$ – $80^{\circ}$  and, therefore, consisted principally of the inactive form. When fractionated with lithium acetate in methyl alcohol into three fractions, each fraction, before recrystallization, melted at  $76^{\circ}$ – $77^{\circ}$  and, when recrystallized from petroleum ether, melted at  $83^{\circ}$ – $84^{\circ}$ . This is the melting point found for the inactive form and reported in a previous paper.

0.1200 gram of the substance gave 0.3330 gram  $\text{CO}_2$  and 0.1350 gram  $\text{H}_2\text{O}$ .

1.7115 grams of the acid dissolved in a mixture of benzene and pure methyl alcohol required 43 cc. of  $\frac{N}{10}$  NaOH for neutralization, using phenolphthalein as an indicator.

0.4802 gram of the acid, as above, required 12.15 cc. of  $\frac{N}{10}$  NaOH for neutralization.

	Calculated for $\text{C}_{26}\text{H}_{40}\text{O}_8$	Found, <sup>a</sup>	
C.....	75.33	75.68	
H.....	12.50	12.59	
M. W.....	398	398	395

### *Salts of Cerebronic Acid.*

**Salts.**—The sodium and lithium salts were prepared and their solubilities studied in order to find the best solvent for purification and also to establish the conditions for the separation of the oxidation product of cerebronic acid from the unchanged acid (see below). The determinations were carried out as follows: The salt was treated with an excess of the boiling solvent; the saturated solution was filtered off, using a hot funnel, and allowed to stand in the ice box over night. The precipitate was filtered off, dried and weighed. The residue from the filtrate was also determined. This gave the total solubility, the amount that could be obtained in recrystallization and the solubility at  $0^{\circ}$ .

**Sodium Salt.**<sup>3</sup>—**SOLUBILITIES.** 140 cc. of boiling methyl alcohol, saturated with the sodium salt, deposited, upon cooling to  $0^{\circ}$ , 1.47 grams. 20 cc. of the filtrate, saturated at  $0^{\circ}$ , gave a residue of 0.09 gram upon evaporation to dryness.

110 cc. of ethyl alcohol, as above, deposited 2.094 grams. 20 cc. of the filtrate gave a residue of 0.032 gram.

<sup>a</sup> Thierfelder: *Zeitschr. f. physiol. Chem.*, xliii, p. 26, 1904–05.

Therefore, 100 cc. of boiling methyl alcohol takes up 1.50 grams of the sodium salt. At 0° it contains 0.45 gram.

100 cc. of boiling ethyl alcohol contains 2.06 grams; at 0°, 0.16 gram.

*Analysis.*—0.1125 gram of the substance gave 0.2931 gram CO<sub>2</sub> and 0.1196 gram H<sub>2</sub>O.

	Calculated for C <sub>25</sub> H <sub>40</sub> O <sub>2</sub> Na.	Found.
C.....	71.19	71.06
H.....	11.72	11.90

*Lithium Salt.*—SOLUBILITIES. 100 cc. of boiling methyl alcohol takes up 3.46 grams of the salt, while at 0° it contains 0.235 gram.

100 cc. of ethyl alcohol at its boiling point takes up 1.42 grams of the salt and at 0° contains 0.39 gram.

*Analysis.*—0.1448 gram substance gave 0.3928 gram CO<sub>2</sub> and 0.1593 gram H<sub>2</sub>O.

	Calculated for C <sub>25</sub> H <sub>40</sub> O <sub>2</sub> Li.	Found.
C.....	74.18	74.00
H.....	12.21	12.31

### *Inactive Ethyl Ester.*

Thudichum<sup>4</sup> describes an ethyl ester of neurostearic acid (cerebronic acid) which he obtained from the hydrolysis of phrenosine (cerebrine) with ethyl alcohol and sulphuric acid. This product melted at 56° but when distilled in vacuum melted at 52°. Since the acid derived from this melted at 84° it must have been the inactive ester. We prepared the ester from the inactive acid as follows: 10 grams of the acid were dissolved in 500 cc. absolute ethyl alcohol, 10 cc. concentrated sulphuric acid added and the mixture heated on the water bath for seven hours. Upon standing over night at 0° the ester almost completely separates out. This was recrystallized from alcohol containing sulphuric acid, then from absolute alcohol and finally from dry acetone. It is very soluble in ethyl acetate, from which it separates in large glistening crystals. The ester melted at 52°–53° and solidified at 51°–52°. For analy-

<sup>4</sup> Thudichum: *Chemical Constitution of the Brain*, p. 162; German edition, p. 195.

sis the product was dried three hours in a chloroform bath over sulphuric acid.

0.1128 gram substance gave 0.3142 gram  $\text{CO}_2$  and 0.1265 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{26}\text{H}_{46}\text{O}_7 \cdot \text{C}_2\text{H}_5$	Found.
C.....	75.79	75.98
H.....	12.55	12.75

*Acetate of the Ethyl Ester*,  $\text{C}_{23}\text{H}_{47}\text{CH}(\text{OCOCH}_3)\text{CO}_2\text{C}_2\text{H}_5$ .

Two grams of the ethyl ester were dissolved in 40 cc. of acetic anhydride and heated to gentle boiling for an hour. Upon cooling the reaction product crystallized out. This was recrystallized from acetone and twice from petroleum ether. It forms colorless crystals, which melt at  $55^\circ$ – $57^\circ$  and solidify at  $53^\circ$ – $55^\circ$ . It was dried in the chloroform bath for analysis.

0.1336 gram of substance gave 0.3630 gram  $\text{CO}_2$  and 0.1457 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{26}\text{H}_{46}\text{O}_4$	Found.
C.....	74.28	74.16
H.....	12.05	12.19

Marie<sup>5</sup> describes a similar compound which was obtained by the action of lead acetate upon the ethyl ester of bromocerotic acid and to which he ascribes the above formula. This melted at  $57^\circ$ – $58^\circ$ . Since then it has been claimed that cerotic acid contains twenty-six carbon atoms; if this is so, then the two compounds are not identical. The question will be investigated further.

### *Inactive Methyl Ester.*

Thierfelder<sup>6</sup> describes a methyl ester of cerebronic acid, which he obtained in the hydrolysis of cerebrine and which gave upon hydrolysis an acid melting at  $100^\circ$ – $101^\circ$ . This ester melted at  $65^\circ$ , and was probably either the active form or a mixture of the active with the inactive. The inactive ester was prepared by boiling a solution of 5 grams of the inactive acid in 500 cc. of absolute methyl

<sup>5</sup> Marie: *Ann. de chim. et de phys.*, (7), vii, p. 228, 1896; *Bull. de la soc. chim.*, xv, p. 577, 1896.

<sup>6</sup> *Zeitschr. f. physiol. Chem.*, xlv, p. 367, 1905.

alcohol, which contained 5 cc. of concentrated sulphuric acid, for five hours. The ester which separated out upon standing over night was recrystallized from methyl alcohol, petroleum ether and finally acetone and formed colorless crystals, which melted at  $59^{\circ}$ – $60^{\circ}$  and solidified at  $57^{\circ}$ – $58^{\circ}$ . It was dried in the chloroform bath for analysis.

0.1120 gram substance gave 0.3096 gram  $\text{CO}_2$  and 0.1281 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{25}\text{H}_{40}\text{O}_7\cdot\text{CH}_3$ .	Found.
C.....	75.65	75.39
H.....	12.71	12.80

### *Acetyl Cerebronic Acid.*

Thierfelder<sup>7</sup> attempted to prepare acetyl cerebronic acid by the action of acetyl chloride upon cerebronic acid; he was not able to isolate the free acid in a crystalline condition, but analyzed it as the sodium salt. By the use of acetic anhydride the reaction is smooth and the product easy of isolation.

Two grams of cerebronic acid and 30 cc. of acetic anhydride were heated under a reflux for two hours. Upon cooling the solution the acetylated acid separated out in a crystalline condition. This was filtered in the ice box, washed with cold alcohol several times to remove the acetic anhydride and twice recrystallized from petroleum ether. It is easily soluble in nearly all organic solvents. Acetyl cerebronic acid is a white crystalline solid, which melts at  $55^{\circ}$ – $56^{\circ}$  and solidifies at  $53^{\circ}$ – $54^{\circ}$ . Since the inactive acid was used, this product is the inactive form. The active form would melt higher.

*Analysis.*—0.1144 gram of the substance gave 0.3090 gram  $\text{CO}_2$  and 0.1240 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{25}\text{H}_{38}\text{O}_8$ .	Found.
C.....	73.57	73.50
H.....	11.90	12.15

*Molecular Weight Estimation.*—0.2776 gram of the substance, dissolved in pure methyl alcohol and benzene, required 6.3 cc. of  $\frac{\text{N}}{10}$  NaOH for neutralization.

<sup>7</sup> *Zeitschr. f. physiol. Chem.*, xliii, p. 27, 1904–05.

	Calculated for $C_{27}H_{40}O_4$	Found.
M. W.....	440	441

*Acetyl Determination.*—0.0908 gram of the dried substance was dissolved in 10 cc. of sodium methylate and 50 cc. of pure methyl alcohol, equal to 113.6 cc. of  $\frac{N}{10}$  HCl, and heated under the reflux for an hour. The reaction product then required 109.8 cc. of  $\frac{N}{10}$  HCl for neutralization. Thus, 3.8 cc. of  $\frac{N}{10}$  NaOH were required in the determination. The amount calculated for the splitting off of one acetyl group and the neutralization of the cerebronic acid formed in the reaction is 4.01 cc. of  $\frac{N}{10}$  NaOH.

	Calculated for $C_{25}H_{36}O_2 \cdot COCH_3$	Found.
COCH <sub>3</sub> .....	9.76	9.27

### *Oxidation of Cerebronic Acid.*

This was carried out according to the directions of Levene and Jacobs. The action takes about two hours on the steam bath; this is better than heating with a free flame, in that bumping and possible foaming are avoided. After the addition of sodium bisulphite, the mixture is acidified, the acids filtered off, dissolved in alcohol and changed into the sodium salts. The salts from 9 grams of cerebronic acid are extracted with a liter of boiling methyl alcohol. The residue is principally the salt of the unchanged cerebronic acid. The acid obtained from the filtrate is transformed into the lithium salt and this extracted with hot alcohol as long as a noticeable precipitate forms upon cooling the filtrate. Usually 1 liter is sufficient. The residue is the lithium salt of the new acid. This is converted into the free acid and purified through the lead salt as described above for cerebronic acid. It melts at 80°–81°. The possible identity of this acid with lignoceric acid has already been mentioned. This will be discussed in a future paper.

0.1196 gram substance gave 0.3428 gram CO<sub>2</sub> and 0.1416 gram H<sub>2</sub>O.

0.5946 gram of the acid, dissolved in methyl alcohol and benzene, required 15.95 cc. of  $\frac{N}{10}$  NaOH for neutralization.

	Calculated for $C_{27}H_{40}O_2$	Found.
C.....	78.20	78.15
H.....	13.20	13.15
M. W.....	368	373

**Sodium Salt.**—SOLUBILITIES. 100 cc. methyl alcohol contain, at its boiling point, 2.186 grams; at 0°, 0.243 gram.

100 cc. ethyl alcohol contains, at its boiling point, 1.870 grams; at 0°, 0.080 gram.

**Analysis.**—0.1004 gram of the substance gave 0.2702 gram CO<sub>2</sub> and 0.1099 gram H<sub>2</sub>O.

	Calculated for C <sub>24</sub> H <sub>48</sub> O <sub>2</sub> Na.	Found.
C.....	73.77	73.40
H.....	12.13	12.25

**Lithium Salt.**—This salt begins to soften at 172° and melts at 173°–178° (not sharp).

SOLUBILITIES. 100 cc. boiling ethyl alcohol contains 0.244 gram; at 0°, 0.06 gram. 100 cc. boiling methyl alcohol contains 0.210 gram and at 0°, 0.095 gram.

**Analysis.**—0.1265 gram of the substance gave 0.3576 gram CO<sub>2</sub> and 0.1405 gram H<sub>2</sub>O.

	Calculated for C <sub>24</sub> H <sub>48</sub> O <sub>2</sub> Li.	und.
C.....	76.92	77.10
H.....	12.65	12.40

### *Reduction of Cerebronic Acid.*

Levene and Jacobs obtained a hydrocarbon which had nearly the required melting point for pentacosan. We have repeated the reduction under slightly different conditions and have obtained a hydrocarbon which shows the melting point given for pentacosan.

Lots of 5 grams of cerebronic acid, 25 cc. of hydriodic acid of specific gravity 1.96 and 2 grams of red phosphorus were heated in sealed tubes at 125°–130° for eight hours. The solid contents of the tubes were filtered off, after diluting the acid with much water. In the first experiments the product was crystallized from alcohol, and then changed into the sodium salt by dissolving in absolute alcohol, neutralizing to phenolphthalein with sodium methylate and evaporating to dryness. The distillation of the sodium salt was accompanied with foaming, however, and so in the later experiments the product once crystallized out of alcohol was thoroughly

dried in vacuum and distilled in the vacuum of a Geryke pump. The reaction was accompanied by decomposition and some foaming; the temperature was about  $320^{\circ}$ . The distillate was taken up in absolute alcohol, neutralized with sodium methylate, evaporated to dryness, and the dry mixture extracted with ether. The residue was changed into the free acid, again distilled and treated as above. The combined ether extract was dried over anhydrous sodium sulphate, the ether removed and the product distilled in vacuum. The distillate was recrystallized from ether or alcohol. It melted at  $53^{\circ}$ – $56^{\circ}$ ; a second sample melted at  $52^{\circ}$ – $55^{\circ}$ . Recrystallized from acetone it showed the same melting point. The product was then distilled, after the addition of a few cubic centimeters of sodium methylate solution and evaporation to dryness, the distillate recrystallized from acetone and thoroughly dried. It then melted at  $53^{\circ}$ – $54^{\circ}$ . According to Marie<sup>8</sup> pentacosan should melt at  $53.5^{\circ}$ – $54^{\circ}$ .

0.1140 gram of the substance gave 0.3566 gram  $\text{CO}_2$  and 0.1444 gram  $\text{H}_2\text{O}$ .

	Calculated for	
	$\text{C}_{24}\text{H}_{48}\text{O}_2$	Found.
C.....	85.13	85.30
H.....	14.89	14.18

### Reduction of $\text{C}_{24}\text{H}_{48}\text{O}_2$ .

The above method was then applied to other fatty acids, in the hope that it might prove a general method for the reduction of acids to hydrocarbons. The result was disappointing.<sup>9</sup> In the case of  $\text{C}_{24}\text{H}_{48}\text{O}_2$ , obtained from the oxidation of cerebronic acid, the product of the distillation was a mixture of a large amount of the ester of the acid with a small amount of the hydrocarbon. The ester was saponified by boiling with alcoholic potash, the solution evaporated to dryness and the mixture extracted with ether. A small

<sup>8</sup> *Bull. de la soc. chim.*, xv, p. 567, 1896.

<sup>9</sup> The reaction was also tried with stearic and palmitic acids. The product in these cases was found to be a mixture of the unchanged acid with the ethyl ester. Thus it is probably the presence of the hydroxyl group which makes the reaction possible in cerebronic acid. It is also evident that the hydroxyl group is reduced simultaneously with or after the carboxyl group, otherwise the normal acid would be obtained.



quantity of hydrocarbon remained. This was recrystallized from alcohol and dried in the chloroform bath. It melted at  $51^{\circ}$ – $52^{\circ}$ . According to Krafft<sup>10</sup>  $C_{24}H_{50}$  should melt at  $51.1^{\circ}$ .

	Calculated for $C_{24}H_{50}$	Found.
C.....	85.10	85.27
H.....	14.90	14.79

<sup>10</sup> *Ber. d. deutsch. chem. Gesellsch.*, xv, p. 1711, 1882.

## ON THE COMPONENTS OF SPHINGOMYELIN.\*

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

The observations reported in this brief communication were made in the course of an investigation into the chemical structure of sphingomyelin. On hydrolysis of this phosphatide two substances were isolated which had not been described by previous workers. One has the nature of an acid, of the composition  $C_{28}H_{47}\cdot COOH$ . It was isolated as the ethyl ester,  $C_{28}H_{47}\cdot COOC_2H_5$ . Two isomers of the substance are known: lignoceric and carnaubic acids. The melting point of the ester seems to indicate that the isolated acid is lignoceric. The correctness of the assumption will have to be corroborated by additional evidence. The melting point of the ester, alone, and also when mixed with the corresponding ester of lignoceric acid, was  $55^{\circ}C$ .

The analysis of the substance revealed the following composition:

	Calculated for $C_{28}H_{47}\cdot COOC_2H_5$ .	Found.
C .....	78.80	78.50
H .....	13.10	13.20

The other substance had the properties of a base and was analyzed as a sulphate. It had the general properties of sphingosin, but differed in its melting point, in its solubility, and in the magnitude of its optical rotation. The substance had the composition  $C_{16}H_{31}NO_2$ , and hence may be regarded as a lower homologue of sphingosin. The melting point of the sulphate is  $225^{\circ}C$ . (uncorrected). The analytical data are the following:

\* Received for publication, May 15, 1913.

*Components of Sphingomyelin.*

	Calculated for (C <sub>15</sub> H <sub>31</sub> NO <sub>2</sub> )H <sub>2</sub> SO <sub>2</sub> .	Found.
C .....	58.80	58.37
H .....	10.45	10.58
N .....	4.58	4.75
S .....	5.22	5.37

Most of the nitrogen of the substance can be liberated in the form of nitrogen gas by means of nitrous acid in the Van Slyke apparatus.

## VISCERAL ORGANISMS.

By ALEXIS CARREL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

In previous articles<sup>1</sup> it was shown that connective tissue could be kept *in vitro* for several months in a condition of active life, and that a fragment of heart could pulsate normally for more than one hundred days after its extirpation from the animal; but the quantity of connective tissue maintained in permanent life was very small. It would be important, for the study of many problems, to keep entire organs alive outside of the organism. Therefore I attempted to develop a technic by which a system of organs could be caused to live *in vitro*.

The method consisted of removing aseptically, *en masse*, heart, lungs, liver, stomach and part of the intestines, pancreas, adrenals, kidneys and spleen of an animal, a cat generally being used, and preserving these organs in an incubator at the temperature of 38 C. (100.4 F.), while the lungs were being artificially ventilated.

The operation comprised five stages:

1. A cat was etherized. The skin of the anterior part of the neck, thorax and abdomen was sterilized. Then the esophagus was ligated and severed under aseptic technic. The trachea was cut transversely and a glass tube inserted in its lumen. A catheter was introduced into it as far as the bifurcation, according to the Meltzer and Auer method.

2. The abdomen was opened. The aorta and vena cava were tied and cut in the lower part of the abdomen. The small intestine was cut under aseptic technic. The ureters were severed. Then the aorta and vena cava were isolated from the posterior walls of the

<sup>1</sup> Carrel, Alexis: Rejuvenation of Cultures of Tissues, THE JOURNAL A. M. A., Nov. 11, 1911, p. 1611; Jour. Exper. Med., 1912, xv, 516. Pozzi, C. R.: Bull. de l'Acad. de méd., Paris, June, 1912.

abdomen and their posterior branches tied. The peritoneum surrounding the kidney was dissected. The splanchnic nerves were cut. Then the abdominal viscera were wrapped in a Japanese silk towel, thus being completely separated from the abdominal wall. They remained united to the animal by the aorta and vena cava.

3. The thoracic cavity was opened and the mammary vessels were clamped. Then the thorax was severed longitudinally, and the diaphragm completely separated from the thoracic wall. Artificial respiration was established. The anonymous arteries were tied, and the animal died. The superior vena cava and the azygos vein were tied and cut. The vagus, sympathetic and phrenic nerves were severed. All the posterior branches of the thoracic aorta were cut. Generally the heart pulsated weakly and the blood-pressure was very low.

4. Then the thoracic and abdominal viscera, united through their blood-vessels, were removed from the cadaver of the animal, and placed in a tray containing Ringer's solution, at about 38 C. Ordinarily the heart still pulsated slowly and regularly. But the blood-pressure was very low, the arterial pulsations weak and the appearance of the organs very anemic. A careful hemostasis of the small vessels was made. After a few minutes the blood-pressure rose and, in a few cases, became almost normal. Generally a blood transfusion was made from the carotid artery of another cat to the inferior vena cava of the visceral organism. Then the lungs became pink, the blood-pressure was higher than normal and the heart was beating strongly from 120 to 150 per minute. The abdominal aorta pulsated violently, and strong pulsations could be seen in the arteries of the stomach, liver and kidneys. Peristaltic contractions of the stomach and of the intestines were observed. If, a few minutes after the transfusion, the pressure was still above normal, a quantity of blood was allowed to flow from the lower part of the abdominal aorta. Then, the appearance of the viscera was the same as the viscera of a normal animal.

5. The visceral organism was placed in a tin box filled with Ringer's solution, covered with thin Japanese silk and protected by a glass cover. The tracheal tube and the esophageal tubes were fastened to proper openings in the anterior wall of the box. Arti-

ficial respiration was carried on by a continuous current of air interrupted ten times per minute. Dr. Meltzer kindly examined the respiratory conditions in these experiments, showing me a method for obtaining a proper ventilation of the lungs. He has contributed, in a large measure, to the rapid success of these experiments. Water or food could be injected into the stomach through the esophageal tube. The intestine was pulled through a glass and rubber tube fixed through the posterior wall of the box. The end of the intestine was fixed by a circular suture to the edge of the rubber tube, an artificial anus being made. The box was then put into an incubator at a constant temperature of about 38 C.

It was then observed that the viscera were living in an apparently normal condition. The pulsations of the heart and the circulation of the organs were normal. The intestine emptied itself through the artificial anus by regular peristaltic contractions. When the intestine was empty, bile and intestinal juices were evacuated. In an experiment in which the stomach was full of meat at the time of death, normal digestion occurred. After five or six hours, hyperemia of the peritoneum of the intestine appeared. It seemed as though peritonitis developed progressively and in some cases the intestine was paralyzed. Nevertheless, the circulation remained excellent and a section of a small mesenteric artery produced an abundant hemorrhage.

Some of the visceral organisms died almost suddenly after three or four hours, but most of them were still in normal condition ten hours and even twelve and thirteen hours after the death of the animal to which the organs belonged. The death of the visceral organism was announced by some irregularities in the pulsations of the heart, which were also weaker. Then the heart stopped suddenly.

In the last experiment the death of the visceral organism occurred thirteen hours and fifteen minutes after the death of the cat from which it originated. It is probable that the duration of life will be increased by certain modifications of the technic; but, as it is, a visceral organism can profitably be used for the study of many physiologic and chemical problems.

## ARTIFICIAL ACTIVATION OF THE GROWTH IN VITRO OF CONNECTIVE TISSUE.\*

By ALEXIS CARREL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

### INTRODUCTION.

If the factors that bring about the multiplication of cells and the growth of tissues were discovered, it would perhaps become possible to activate artificially the processes of repair. Then aseptic wounds could probably be caused to cicatrize more rapidly. If the rate of the reparation of tissues were activated ten times only, a cutaneous wound would heal in less than twenty-four hours, and a fracture of the leg would be cured in four or five days. It is permissible to think that this hypothesis is not unreasonable. Jacques Loeb, in his fundamental experiments on artificial parthogenesis, has demonstrated that cell division can be induced by slight changes in the composition of the sea water in which the sea urchin's eggs are placed. It might even be supposed that certain modifications of the *milieu intérieur* of the tissues of mammals would bring about the multiplication of their cells. In 1907 and 1908 I began, therefore, to study the processes of reparation of small cutaneous wounds and the action of a great many substances on the rate of their cicatrization. It was found that the proliferation of epithelium and of connective tissues was activated under certain conditions by dressings made with the pulp of tissues and organs. For instance, thyroid gland pulp deposited on cutaneous wounds of the dog brought about the formation of exuberant granulations. Applied to bones, it produced a marked thickening of the periosteum. The external coat of an artery preserved in cold storage in a mixture of thyroid gland and Locke's solution, and transplanted afterwards into a dog's carotid, underwent an enormous hypertrophy. How-

\* Received for publication, June 6, 1912.

ever, it was difficult to study with precision the influence of these substances on the tissues of living dogs. It became evident that the changes brought about by them could be more precisely observed if the tissues were isolated from the organism and made to live in a medium of known composition. Therefore, I undertook to adapt to the cultivation of mammalian tissues the method used by Harrison in his researches on the growth of the central nervous system of frog embryos in a drop of lymph. In some experiments that I made in 1911 with the collaboration of Dr. Burrows, it appeared that the growth of chicken tissues were activated when extracts of the Rous chicken sarcoma and of chick embryo were added to the culture medium.

In 1912, by using a more precise technique, I was able to study quantitatively the influence of tissue juices on the growth *in vitro* of connective tissue and some of the characteristics of their activating power.

#### METHOD.

The method consists in measuring the extent of the growth of fragments of tissues placed in normal plasma and in plasma containing a known quantity of a tissue juice. The extracts were made of chick embryos from six to twenty days old, of spleen, kidney, muscle, etc. of the adult chicken, of the Rous sarcoma, of thyroid gland, spleen, and muscle of the adult dog, and of spleen of the adult rabbit. The tissues were cut into very small fragments, or cut and ground with sand in a mortar, or cut, ground, and frozen in ice and salt, and then put for a short time in the incubator at 38° C. Afterwards, to one volume of tissue were added from one third of a volume to four or five volumes of Ringer solution. The tubes containing the mixtures were put in cold storage. After a period varying from a few minutes to twenty days, the tubes were centrifugalized. The supernatant fluid was used pure or diluted with Ringer solution. In some experiments the fluid was heated at 56° or 70° C. for ten, twenty, or thirty-five minutes. In other experiments, it was filtered through paper or through a Berkefeld or a Chamberland filter.



The culture medium was composed of one volume of extract and two volumes of hypotonic plasma. The hypotonic plasma was obtained by adding two volumes of distilled water to three volumes of normal plasma.

The majority of the experiments were performed on hearts of chick embryos from seven to fifteen days old. The ventricular wall was divided into small fragments almost identical in size. The fragments, placed in the culture medium, were rapidly surrounded by a dense ring of connective tissue cells. Fragments of skin were also used. In the experiments performed on dog's tissues, fragments of periosteum were employed. With each experiment control cultures in normal hypotonic plasma were made. In order that the results should be comparable, the cultures were prepared with extreme care and precision.

The specimens were examined after twenty-four, forty-eight, and seventy-two hours. The area of the new issue was calculated accurately after the diameter of the original fragment and the width of the ring of new tissue had been measured by the micrometer. But the thickness of the new tissue could not be known exactly. In all the experiments where extracts were added to the medium, the growth was not only more extensive but also denser than in the cultures which contained no extracts. The increase in thickness was generally not considered in the calculation of the value of the acceleration of the growth, this calculation being based only on the increase in area of the tissue. The increase of the tissues was really greater therefore, than appears in the description of the results.

#### RESULTS.

In every experiment the fragments of heart, skin, and periosteum, cultivated in plasma containing an extract, grew more rapidly than their controls. It is certain, then, that tissue juices have the power to activate *in vitro* the growth of connective tissue.

The value of this power varied according to the method used in the preparation of the extract. An extract obtained by the centrifugalization of embryonic tissue, a few minutes after it had been mixed with Ringer solution, increased the rate of growth two and

a half and three times. The acceleration of the growth was much more marked when the mixture of tissues and Ringer solution was allowed to stand in the refrigerator for twenty-four hours or several days before being centrifugalized. For instance, in experiment 1,734, Ringer solution containing embryonic pulp had been preserved for twenty days in cold storage before being centrifugalized. In twenty-four hours, the area of new connective tissue was thirty times larger in the cultures containing the extract than in the controls. The extracts of tissues cut in small fragments, mashed, and frozen, were generally very active. The experiments made with these different extracts showed that they were able to increase the growth of connective tissue from about three to forty times.

1. *Effect of Concentration of an Extract on Its Activating Power.*—An extract diluted with Ringer solution was less active than one that was not diluted. For instance, in experiment 1,577, fragments of heart were cultivated in plasma containing  $\frac{1}{8}$ ,  $\frac{1}{6}$ ,  $\frac{1}{12}$ ,  $\frac{1}{24}$ , and  $\frac{1}{48}$  of embryonal juice dissolved in Ringer solution. The control cultures were made with plasma containing a like quantity of Ringer solution. The areas of connective tissue produced in forty-eight hours were respectively 60, 21, 12, 8.25, and 5, in the experiments, and 5 in the controls. In other experiments the quantity of new connective tissue varied also in direct ratio to the concentration of the extract.

2. *Effect of the Nature of the Tissues on the Activating Power.*—The experiments were performed on extracts of chick embryo from six to twenty days old, of spleen, liver, connective tissue, kidney, heart, and blood corpuscles of the adult chicken, of the Rous sarcoma, and of thyroid gland and muscle of the adult dog.

All the extracts activated the growth of the connective tissue. But the degree of acceleration varied in large measure. Embryonal tissue extract was the most active. Extracts of adult spleen and the Rous sarcoma were almost as active as the extract of chick embryos. Kidney and heart extracts were much less active, while the extracts of connective tissue and of blood corpuscles brought about a slight acceleration only of the growth.

The influence of the extracts of thyroid gland and muscle of the dog on the growth of periosteum was very marked, but thyroid extract was more active than muscle extract.

3. *Effect of the Origin of the Tissues.*—The power of an extract of animal tissues seemed to be specific and was confined to the tissues of another animal of the same species. For instance, the extract of chicken spleen activated greatly the growth of connective tissue of a fragment of the heart of chick embryo, while the activating influence of extracts of dog and rabbit spleen on chick tissue was very slight.

4. *Effect of Heat on the Activating Influence of the Extracts.*—The embryonic extracts began to lose their activating power when they were heated at 56° C. for ten minutes. The diminution was more marked when the extracts were heated for thirty minutes. For instance, in experiment 1,565 an embryonic extract heated at 56° C. for ten minutes lost one third of its power. The same extract heated at 56° C. for thirty-five minutes lost two thirds of its power. An extract of the Rous sarcoma heated at 56° C. for ten minutes lost also one third of its activity. The extracts of adult spleen were slightly modified by the heating at 56° C. for ten minutes. Their activating power remained generally unaltered.

The extracts of adult spleen, of the Rous sarcoma, and of chick embryo, heated for ten minutes at 70° C. lost completely their activating power.

5. *Effect of Filtration on the Activating Power of the Extracts.*—The experiments were made on extracts of chick embryo and of the Rous sarcoma, diluted with Ringer solution.

The power of the extracts was not modified by filtering them through filter paper. However, if the extract contained much cellular debris, the filtration increased its power slightly.

Extracts which were filtered through a Berkefeld filter always lost a great part of their activating power. The area of new connective tissue in cultures to which such a filtered extract had been added was about one third or one fourth smaller than in the cultures containing an unfiltered extract.

Filtration through a Chamberland filter suppressed completely the activating power of an extract. The heart fragments did not

produce more connective tissue in plasma containing such a filtered extract than in plasma containing an equal quantity of Ringer solution. For instance, in experiment 1,538 the areas covered by the new connective tissue were respectively 77, 32, 21, and 21, in media containing (1) unfiltered extract of the Rous sarcoma, (2) extract filtered through a Berkefeld filter, (3) extract filtered through a Chamberland filter, and (4) Ringer solution.

#### SUMMARY AND CONCLUSIONS.

The experiments have shown that extracts of tissues and tissue juices, under certain conditions, accelerate the growth *in vitro* of the connective tissue from about three to forty times. This activating power was found in many tissues. It was much more marked, however, with the extracts of embryos, of adult spleen, and of the Rous sarcoma. The power diminished directly with the dilution of the extracts, and appeared not to apply to the tissues of a heterologous animal. The power was reduced when heated at 56° C., and removed when heated at 70° C. It was diminished markedly by filtration through a Berkefeld filter and was completely suppressed by filtration through a Chamberland filter.

Possibly the finding of the activating power of tissue extracts will have no immediate practical application. Nevertheless, it may be indirectly useful by leading to the discovery of some of the factors determining the growth of tissues and of the unknown laws of cell dynamics, and may ultimately throw light on the mechanism of the cicatrization of wounds.

## STUDIES OF THE DEGENERATION AND REGENERATION OF AXIS CYLINDERS IN VITRO.\*

By RAGNVALD INGEBRIGTSEN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

PLATES 28 TO 40.

The experiments of Harrison,<sup>1</sup> who cultivated the medullary cord of the embryonal frog in coagulated frog lymph, showed that from the neuroblasts of His hyaline threads grew out which increased in length by means of ameboid movements. Harrison has interpreted these threads as axis cylinders. Burrows observed the same phenomenon in cultures in chick plasma of the medullary cord of a chick embryo two days old, and succeeded in staining the newly formed axis cylinder.<sup>2</sup> Lewis and Lewis<sup>3</sup> have described the growth of sympathetic nerves from the intestines of chick embryos in saline solutions.<sup>4</sup>

I began the experiments, the preliminary results of which are given in this publication, with the cultivation in coagulated chick plasma of the neural tube of chick embryos two to three days old, and in a few cases (5 out of 102 experiments) I observed that after ten to twenty-four hours there grew out from the transplanted piece slender filaments with a bulbular end which, by ameboid movements through the plasma, increased the length of the thread. Besides, there occurred in all the cultures a rich proliferation of the

\* Received for publication, November 2, 1912.

<sup>1</sup> Harrison, R. G., *Jour. Exper. Zool.*, 1910, ix, 787; *Anat. Rec.*, 1912, vi, 181.

<sup>2</sup> Burrows, M. T., *Jour. Exper. Zool.*, 1911, x, 63.

<sup>3</sup> Lewis, W. H., and Lewis, M. R., *Anat. Rec.*, 1912, vi, 7, 207.

<sup>4</sup> Since writing this paper, an article by Marinesco and Minca (*Essai de culture des ganglions spinaux de mammifères in vitro*, *Anat. Ans.*, 1912, xlii, 161) has come to my attention. These authors have cultivated spinal ganglia of young cats and rabbits in plasma in large dishes. They observed growth of connective tissue cells and of axis cylinders, which were stained according to the method of Cajal. The newly formed nerve fibers are described in some cases as prolongations of the cell body ending in swellings, in other cases as pericellular networks and plexuses.

embryonal connective tissue cells, and this was probably the cause of the scant percentage of successful cultures, for we know that the growth of connective tissue prevents the development of tissues of a higher order.

I began, therefore, to cultivate the tissues of the central nervous system of chick embryos in a more advanced and differentiated stage and found that pieces of the cerebral cortex of chick embryos six to seven days old gave 100 per cent. of successful cultures as far as the growth of the axis cylinders is concerned.

The cultures were prepared in the ordinary way as described for cultivation of tissues in general by Carrel and Burrows. The embryo was taken out of the egg and placed in Ringer solution heated to 40° C. From the forebrain the covering membranes were removed by means of scissors and forceps, and then, with a cataract knife, very thin slices of the cortex, about one millimeter square, were taken out. These slices were put immediately at room temperature in a drop of plasma on a cover-glass, and the cover-glass was inverted over a hollow slide, without waiting for the coagulation of the plasma. In some cases the plasma was diluted one third or one fourth with distilled water. In other cases it was not diluted. In this way it was possible to prepare each culture in one half or one quarter of a minute. In some cases the tissues were placed on a piece of silk veil in the plasma, the silk being a support for the growing filaments. I shall now describe the phenomena of growth that occur in a culture of the brain of the chick embryo six to seven days old.

After incubating the cultures for ten, eighteen, and twenty-four hours, a number of thin, slender, light-breaking filaments consisting of hyaline protoplasm without granules or visible structure were seen growing out from the pieces. Most of these filaments were of the same thickness, about one half to one micron. Throughout their length they were curved and bent in several directions. After twenty-four hours, most of them reached a length of 250 to 300 microns, and a few even 460 microns. Several of these filaments ended in small swellings of the thread (figure 1). This bulb was sometimes regular and ovoid, and measured two to three microns in width. Some of the filaments ended in a point, and a few had

finger-like branches in which ameboid movements could be observed. These filaments might be present in small numbers in each culture, or they might grow so densely that they formed a real tissue around the fragment of brain. A few of them had branched by the twenty-fourth hour.

After forty-eight hours the fibers increased in length and thickness. Some of the fibers now reached a length of about one millimeter, and one of them was 1.1 millimeters. Some of the thickest fibers showed a plain fibrillation in their central parts, and further in their course swellings were seen. These swellings were of different size and shape. Most of them were slight and somewhat irregular, but in some cases they resembled very much the end bulb of the filament. They consisted always of hyaline protoplasm. Branching of the thickest fibers was frequently observed at this stage, and anastomoses between different branches occurred in some places (figure 2). After forty-eight hours another feature of interest was observed, that is, the occurrence of isolated cells lying free in the plasma near the border of the fragment of tissue. These cells were of two kinds. (1) Large cells, without any definite cell membrane, with many granules, and one large, clear nucleus. They sometimes have one or two short protoplasmatic, filamentary processes, and in a few cases I observed one or two of the above mentioned long protoplasmatic threads with bulbular ends growing out from these cells (figures 3 and 4). (2) Cells of another type which were mostly oblong, sometimes fusiform with sharp outlines. These cells had a very definite cell membrane, one nucleus, and powerful, straight, tapering outgrowths, three to five microns wide, in which granules were visible. The outgrowths never had bulbular endings and were easily distinguished from the above mentioned slender curved hyaline filaments (figure 5). There is no reason to doubt that the cells of this second type are connective tissue cells (glia (?), endothelial (?)), while the first type is similar in character to ganglion cells, and its outgrowths are very much like the filament described by Harrison in the cultures of embryonal frog, and in all probability are axis cylinders.

On the third day there was still some increase in size of these nerve fibers and the longest ones observed were 1.25 millimeters in length.

On the fourth day but little difference was seen. Granulation occurred in some of the fibers and their outlines became less definite. On the fifth and sixth days the fibers disappeared completely.

I continued to work with the brains of chick embryos of different ages. As far as the growth of nerve fibers was concerned a high percentage of the cultures was successful. A few cultures were discarded as not useful on account of technical errors.

TABLE I.  
*Cultures of Brain of Chick Embryo of Different Ages.*

Age of the embryos.	No. of animals employed.	Total No. of cultures.	No. of cultures in which axis cylinders were present.	Percentage of growth of axis cylinders.
2 days	4	19	2	10.5
3 days	10	83	3	3.6
6-10 days	5	32	32	100.0
11-15 days	6	65	40	75.3
16-21 days	8	53	37	69.8
		252	123	

The growth of the axis cylinder from brains of chick embryo of a more advanced age showed practically the same characteristics as described formerly for the six to seven day old embryos. There were no striking differences in the rate of growth. In one of the cultures, there occurred a phenomenon which deserved closer attention. The culture was from the brain of a chick embryo eighteen days old (figure 6). On the third day there had developed a great number of thick and thin axis cylinders which branched and anastomosed, forming a network. The swellings of these fibers were larger than any observed earlier. They appeared like refractive homogeneous globes, four to five microns in diameter, lying in the course of the filament or at the anastomosis between two or three filaments. Sometimes they were oblong, and in shape were very much like bipolar ganglia cells. As they had no nucleus, however, I am sure they were not. I consider them as accumulations of protoplasm analogous to that in the end bulb, and like the latter they give rise to outgrowths of new fibers.

Having established these facts I tried in some few cases to cultivate the spinal cord of chick embryos and succeeded in observing



## 354 *Degeneration and Regeneration of Axis Cylinders in Vitro.*

axis cylinders sprouting out from the pieces of tissue (figure 7). Next I began the cultivation of the cerebral nervous systems of young mammals. Rabbits, cats, and dogs were employed, and preparations were made from the brain, the cerebellum, the spinal cord, and the spinal ganglia. The animals were operated upon under ether anesthesia. The tissue was put directly from the animal into Ringer solution at 40° C., and was then cut into small pieces. The preparation of each culture took about half a minute. A little more time was required for the dissection and cutting of the spinal ganglia with their tough connective tissue envelopes.

TABLE II.

*Cultures of the Central Nervous System of a Rabbit Two Months Old.*

No. of animals employed.	Origin of cultures.	Total No. of cultures.	No. of cultures in which axis cylinders were present.
2	Brain	8	2
	Spinal cord	14	1
	Spinal ganglia	5	0

TABLE III.

*Cultures of the Central Nervous System of a Rabbit Seven Months Old.*

No. of animals employed.	Origin of cultures.	Total No. of cultures.	No. of cultures in which axis cylinders were present.
3	Brain	23	0
	Spinal cord	4	0
	Spinal ganglia	4	2

TABLE IV.

*Cultures of the Central Nervous System of a Cat Six Weeks Old.*

No. of animals employed.	Origin of cultures.	Total No. of cultures.	No. of cultures in which axis cylinders were present.
3	Brain	24	3
	Spinal cord	7	1
	Spinal ganglia	12	2

The development of the axis cylinder in cultures from these young animals was slower than in those from embryonic nervous tissue. They were observed usually after thirty-six or forty-eight hours, and only a few were present in each culture. They showed,

however, the same main characteristics as those from embryonic chicks. The filaments were curved, hyaline, ended usually in a bulb, had branches, and were longitudinally striated when they had reached a certain size. In some of them, especially in those prepared from the gray substance of the spinal cord and from the spinal ganglia, granulations were visible (figures 8 and 9).

The cultures prepared from the brain of these animals showed a development of connective tissue cells only after three days, and, fortunately for the axis cylinders, only a few connective tissue cells appeared in each of the cultures, while the spinal ganglia gave rise to a rich growth of connective tissue cells.

The cultures of the nervous system from rabbits were kept for observation for only three days and a half, were then recorded as positive or negative, and the negative ones were discarded. It is possible, as was learned later, that the results obtained might have been changed and might have shown a greater number of positive cultures if they had been kept for observation for five or six days. In adult animals it is necessary to observe the cultures for a long time to be sure that they will not develop axis cylinders. If they do develop, they live for only a very short time. I have never seen axis cylinders which did not undergo degeneration after the seventh day of the incubation of the culture at 40° C.

I had, however, not yet reached my goal, which was to observe the development of axis cylinders in cultures of the cerebral nervous system of mammals in such a large percentage of the cases that it might be useful for the study of degeneration and regeneration. I succeeded when I began to work with dogs. From the brain of a dog three weeks old I observed a growth of axis cylinders. This growth took place from the cerebrum, but still more abundantly from the cerebellum. Of fifteen cultures prepared from the brain of a dog three weeks old, eleven were positive, and most of these showed an abundance of axis cylinders (figure 10). Out of ten cultures prepared from the cerebellum of two such dogs, every one showed a great number of axis cylinders; that is, 100 per cent. of the cultures were successful.

After twenty-four hours the axis cylinders from the brain of this dog had already appeared, and those from the cerebrum showed

the characteristics mentioned above for the chick, cat, and rabbit. The growth from the cerebellum I will mention a little more particularly (figure 11). These nerve fibers have usually a big bulb at their ends and are bent in curves and angles. They are richly provided with swellings and form branches and anastomoses within the first twenty-four hours. After two or three days the fibers were rather thick (from two to three microns), and some of them showed a faint fibrillation. The swellings now have become very numerous and have a diameter of four to five microns; they are either globular or oblong and are quite homogeneous. After five days' incubation the length of these fibers was 0.9 of a millimeter.

On the fifth day these fibers showed the following degenerative changes: A faint granulation appeared first in the swellings and shortly afterward in the fibers. The outlines of the fibers became less definite, the fibers and the swellings became less refractive, and the edges of the globular swellings appeared as if they had been nibbled. The change was seen at the same time throughout the whole length of the fiber and increased during the next two or three days, so that on the seventh day after incubation all the fibers appeared degenerated.

#### EXPERIMENTAL PART.

I shall describe in detail one of the experiments in which I cut some of the nerve fibers in the cultures of the cerebellum of dogs three weeks old.

After twenty-four hours a large number of axis cylinders had developed, which increased in size during the following two days. On the fourth day I cut through the plasma and some of the nerve fibers contained in it close to the piece of tissue (figure 12). As the coagulated plasmatic jelly is under tension the cut in it produced a small gap by the retraction of the medium. This was immediately filled with fresh plasma from the same animal, which had been kept on ice, and which now coagulated. The cover-slip was again inverted on a hollow slide and incubated. It was observed from hour to hour until late in the evening. The cutting of the plasma had disturbed slightly the central part of the cut nerve fibers, as the retraction had taken place especially at this side. It was observed immedi-

ately after section that the central part of the nerve fibers had been a little crumpled and their outlines were more curved than before (figure 12). This was not true of the peripheral part in which no visible changes took place either at the time of the cutting or in the following nine hours. The next morning, however, that is, twenty hours after the cutting, changes of a different kind had occurred, degenerative as well as regenerative (figure 13). The cut fibers were thinner than on the previous day. This could be determined by comparing them with nearby fibers which were not cut, and which before section were of the same thickness. Moreover, the cut fibers had a faint granulation and the edge of the swellings appeared moth-eaten. The appearance of the central part of the cut fibers had not changed visibly. But in the gap that was filled with plasma there had grown out from the central part of the cut fibers three axis cylinders, of which the longest one, grown during the lapse of twenty hours since the cutting, had a length of 150 microns. There was nothing else extraordinary about them. They looked like the other young axis cylinders, had end bulbs and some swellings. Their growth was followed during the next two days, during which time one of them increased sixty-five microns in length, the others not quite so much (figure 14).

Meanwhile the degeneration in the peripheral part of the cut fibers continued slowly throughout its entire length (figures 14 and 15). The fibers became thinner and less refractive, and the granulations increased. At last, on the sixth day, they could hardly be seen. At this time the other fibers which had not been cut were partly granular but their outlines were still distinct (figure 15). The specimens were stained after three and six days.

The technique which after several attempts was found to give the most successful preparation was the following:

1. Fixation in 2 per cent. formalin for 15 to 18 hours.
2. Staining in a very dilute solution of Held's molybdic acid hematoxylin (6 to 8 drops to 15 c.c. of water) for 12 hours.
3. Differentiation in a dilute solution of the Weigert borax-potassium-ferrocyanide solution (2 drops to 20 c.c. of water) for 12 to 20 hours.
4. Dehydration, xylol, Canada balsam.

The color of the fibers is greyish blue, and they are very distinct. The method seems to injure the fibers a little, for even when fixed

and stained after three days' incubation the stained specimens are plainly granular, while the living fibers show no granulation at all. I have, therefore, studied mainly the living specimens, and most of the illustrations given here are camera lucida drawings of living fibers. The camera lucida was employed because it is hardly possible to get a photograph of one nerve fiber in its whole length, as it curves and bends through many planes, and living fibers were drawn because these are of course of greater interest than those that are dead.

#### CONCLUSIONS.

1. The brains of chick embryos, of cats six weeks old, of rabbits two months old, and of dogs three weeks old, when cultivated *in vitro*, develop long filaments which, according to their growth and their anatomical and tinctorial characters, must be considered as true axis cylinders.

2. Similar structures develop from spinal ganglia of rabbits seven months old, and from the spinal cord of cats six weeks old, and of rabbits two months old.

3. When severed from their origin by section these threads undergo degenerative changes which do not appear after nine hours, but which are seen after twenty hours, and continue until in the course of the following two days the thread degenerates completely.

4. After twenty hours the development of new axis cylinders from the central part of the cut fibers is observed.

#### EXPLANATION OF PLATES.

##### PLATE 28.

FIG. 1. Culture of brain cortex twenty-four hours old from a chick embryo six days old. Culture on silk veil.

##### PLATE 29.

FIG. 2. Culture of brain cortex two days old from a chick embryo fourteen days old.

##### PLATE 30.

FIG. 3. Culture of brain cortex forty-eight hours old from a chick embryo six days old. Isolated cells, one of them with an axis cylinder.

##### PLATE 31.

FIG. 4. Culture of brain cortex forty-eight hours old from a chick embryo twelve days old. Isolated ganglion cell with axis cylinder.



FIG. 1.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders *in Vitro*.)



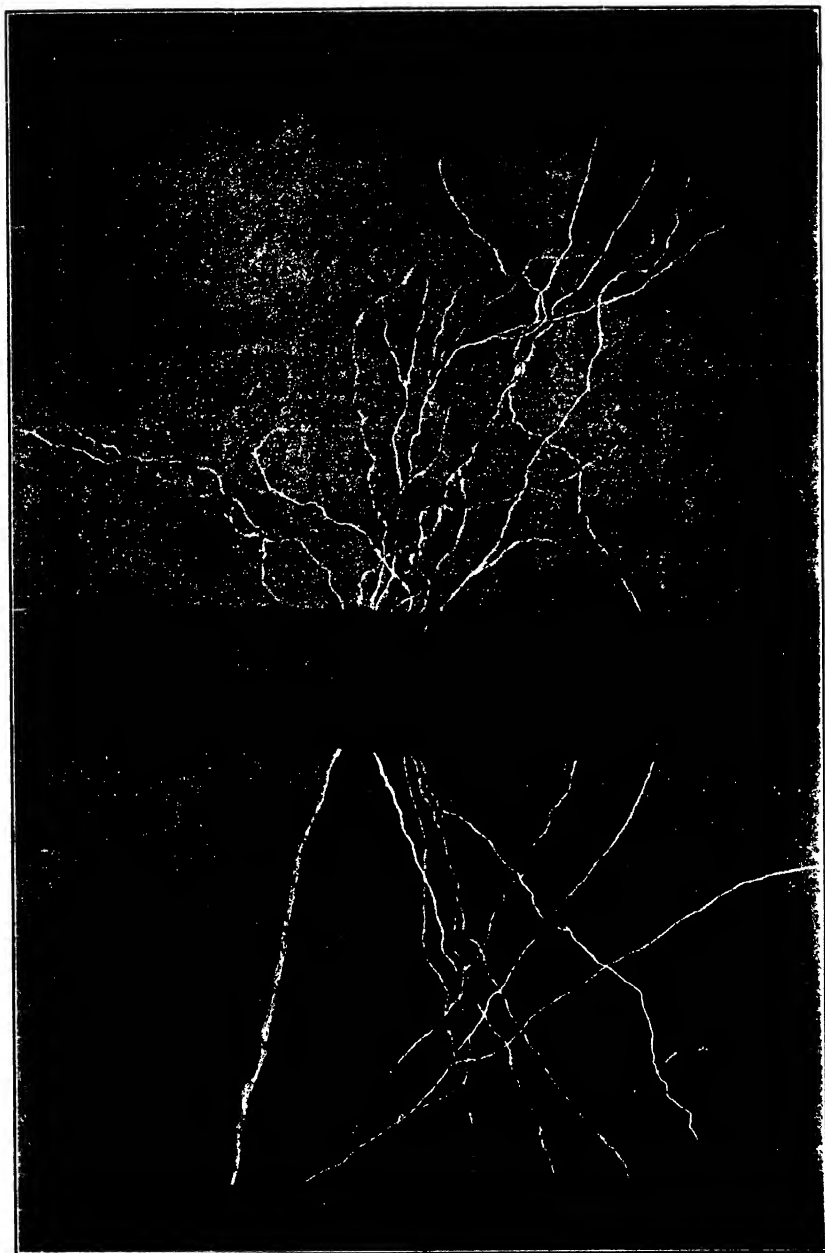


FIG. 2.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders *in Vitro*.)





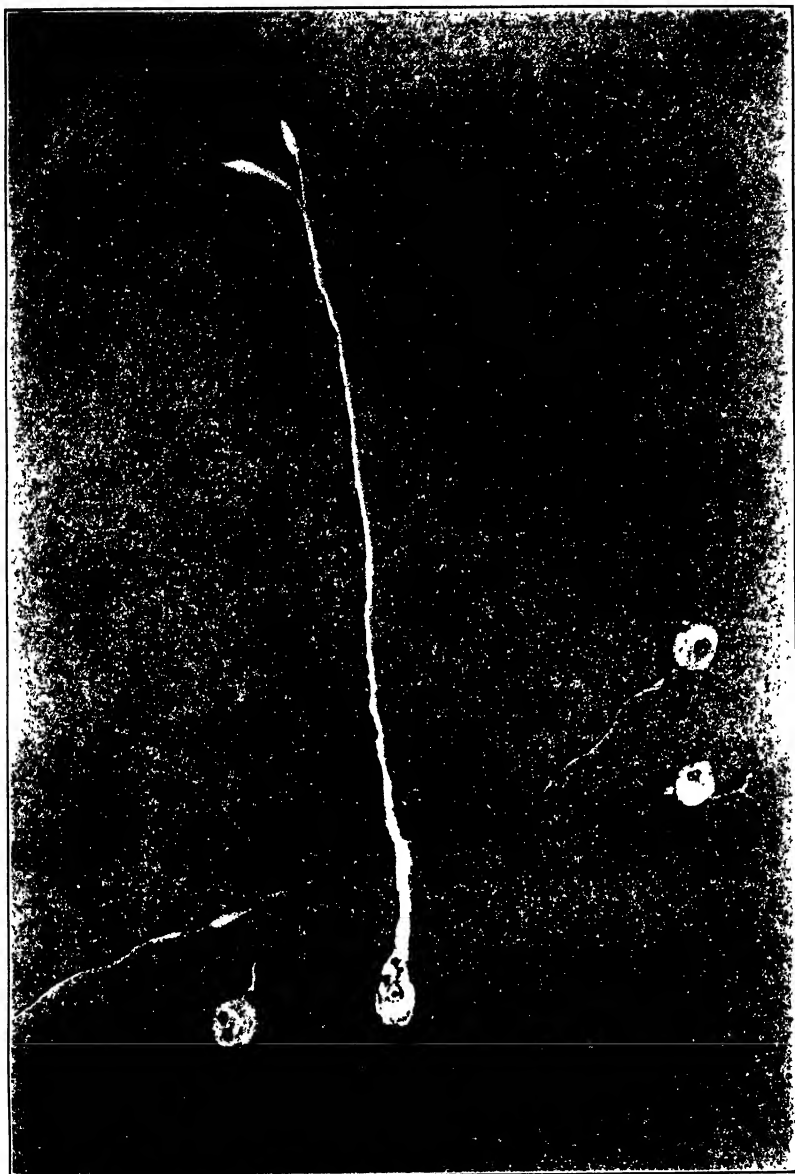


FIG. 3.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders *in Vitro*.)



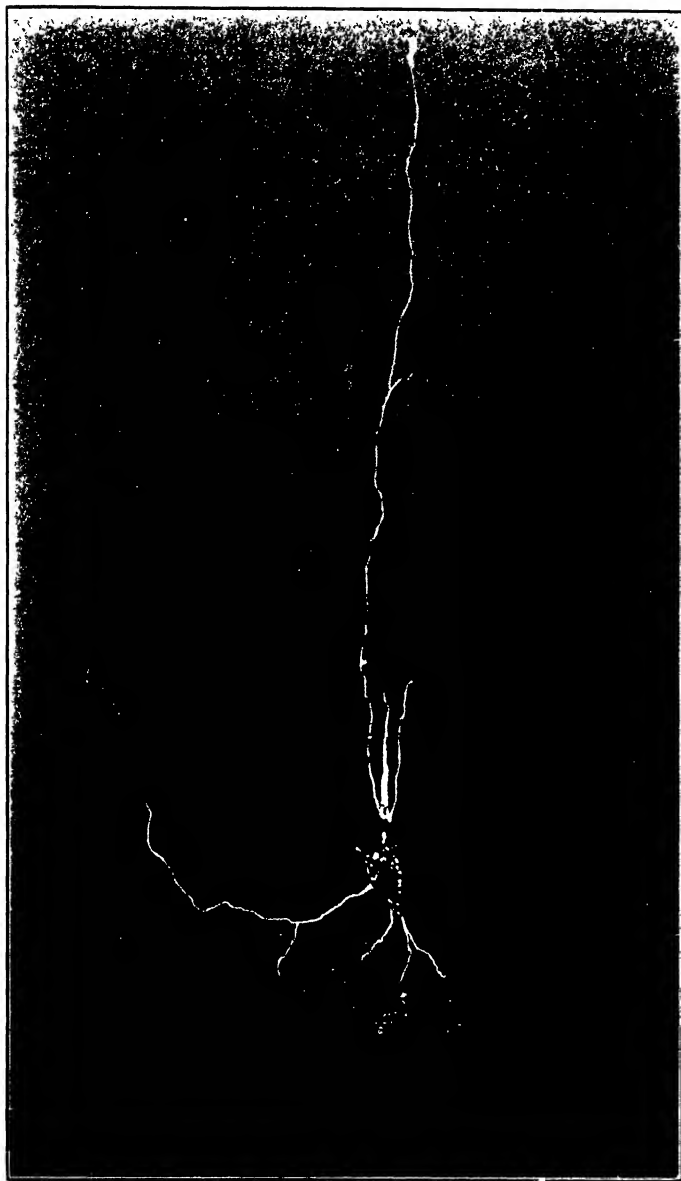


FIG. 4.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders *in Vitro*.)





FIG. 5.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders *in Vitro*.)



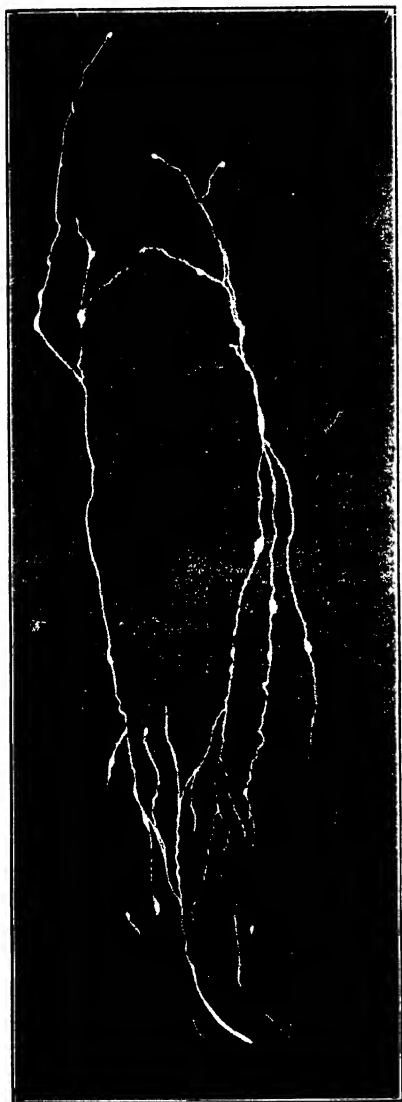


FIG. 6.

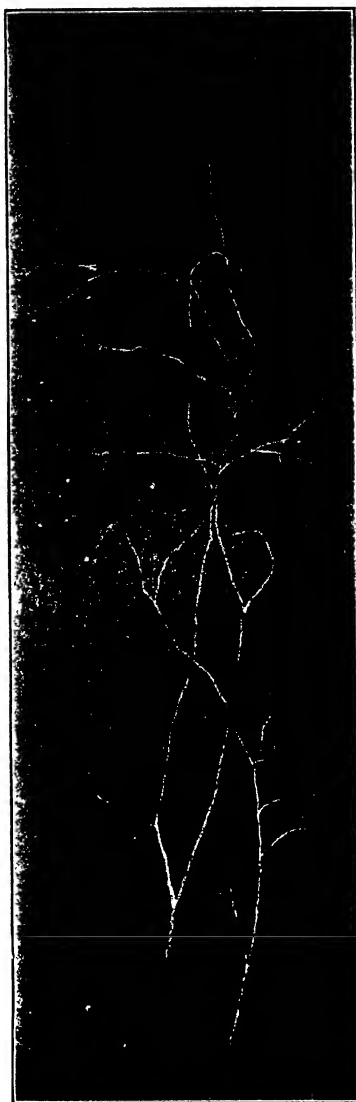


FIG. 7.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders *in Vitro*.)







FIG. 8.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders *in Vitro*.)



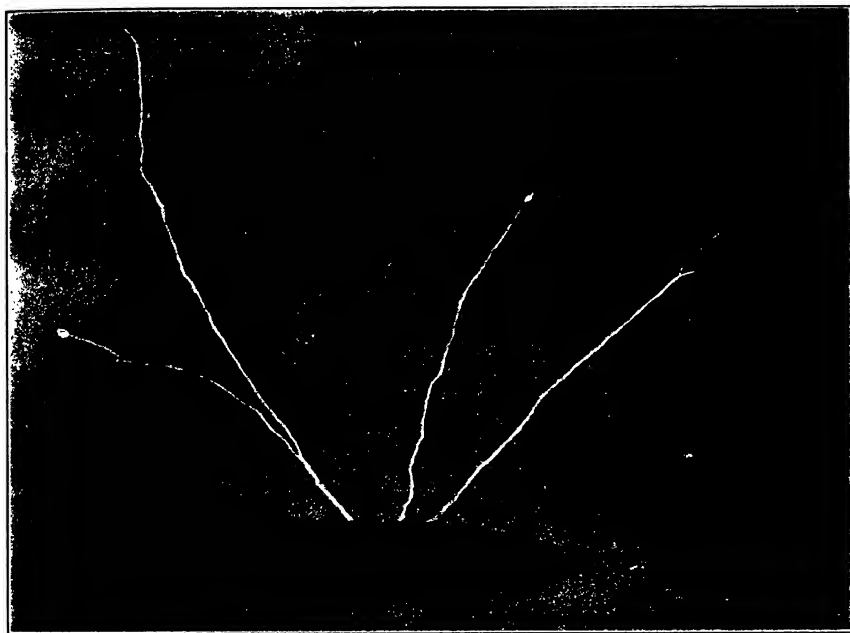


FIG. 9.

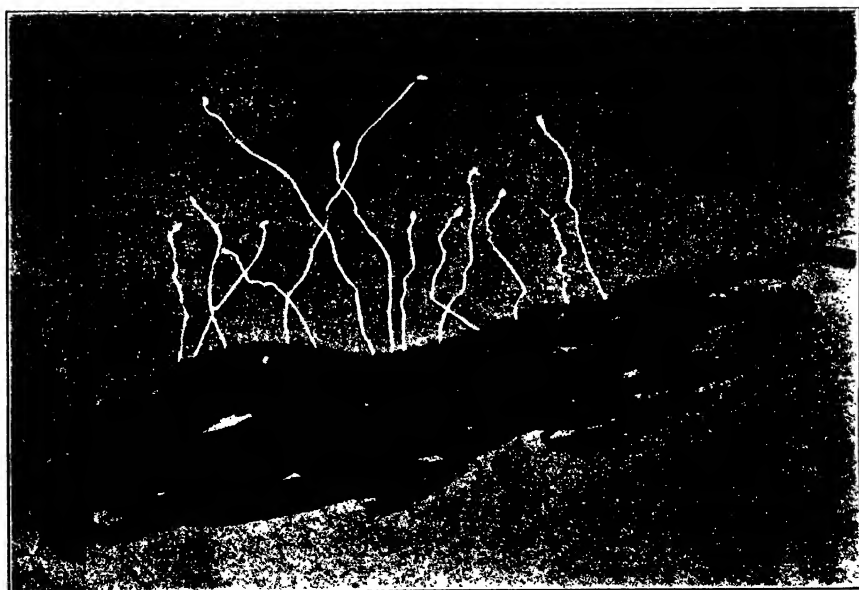


FIG. 10.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders *in Vitro*.)

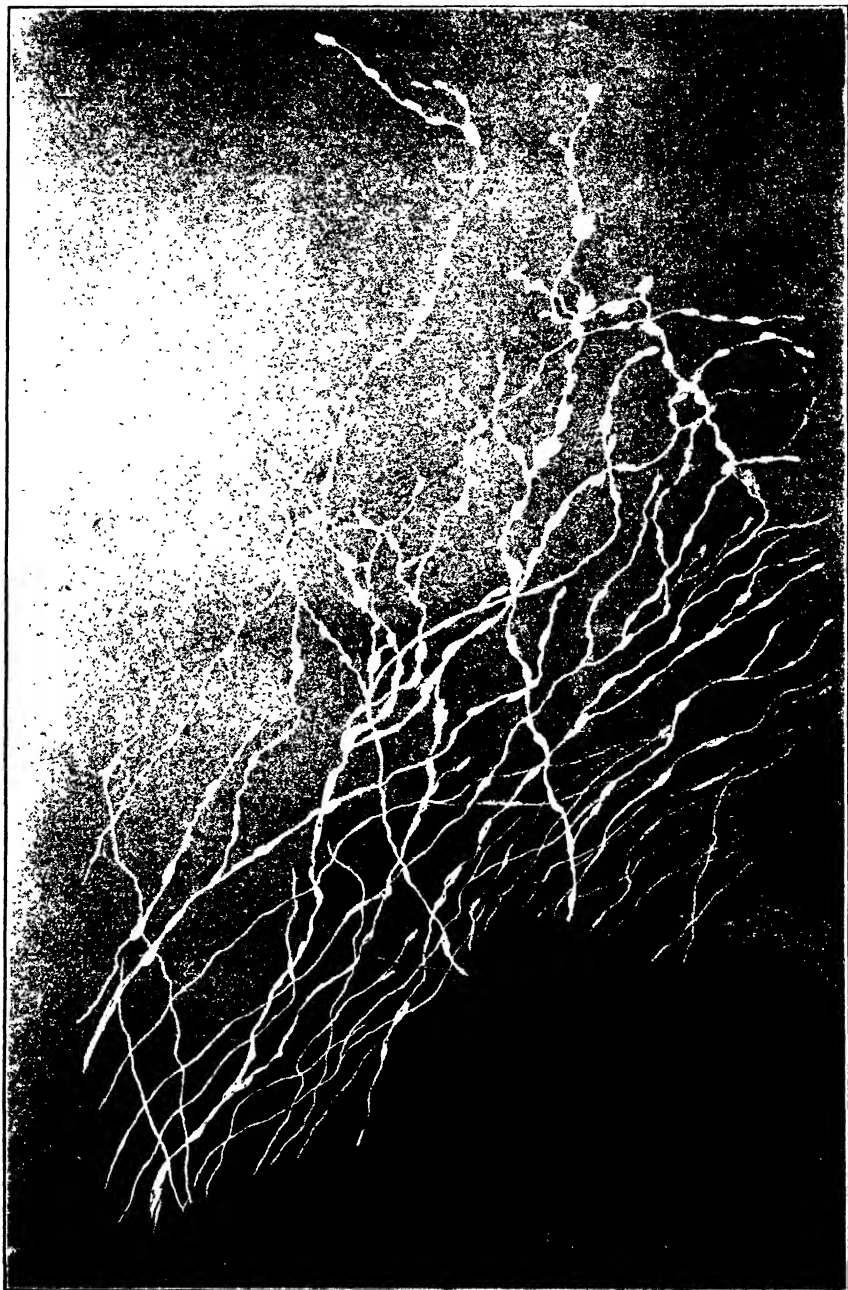


FIG. 11.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders *in Vitro*.)

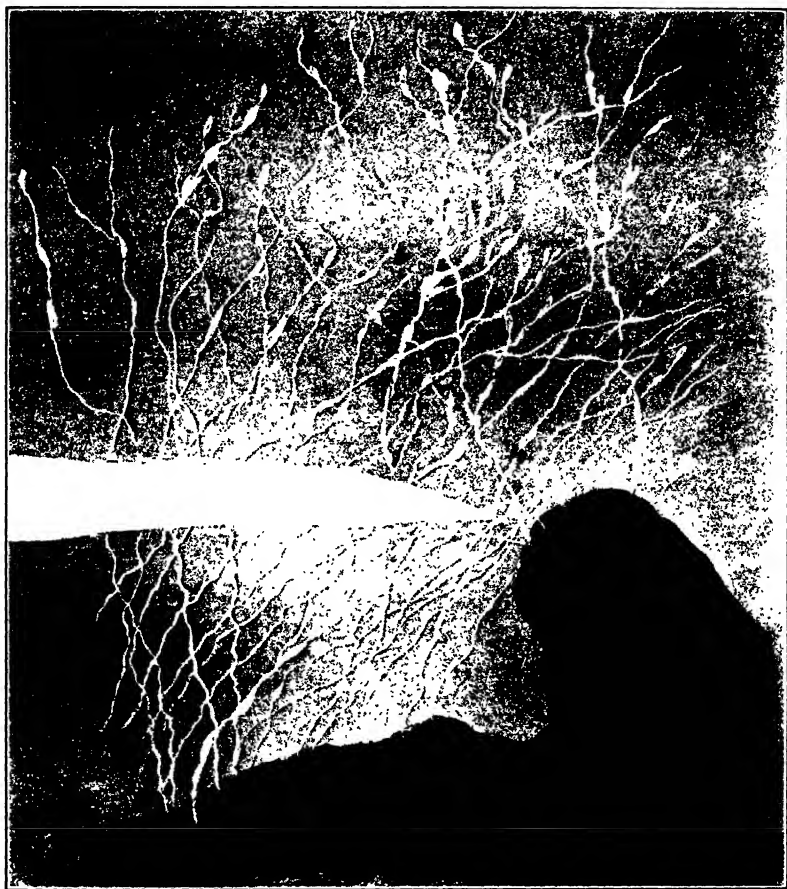


FIG. 12.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders *in Vitro*.)



FIG. 13.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders *in Vitro*.)

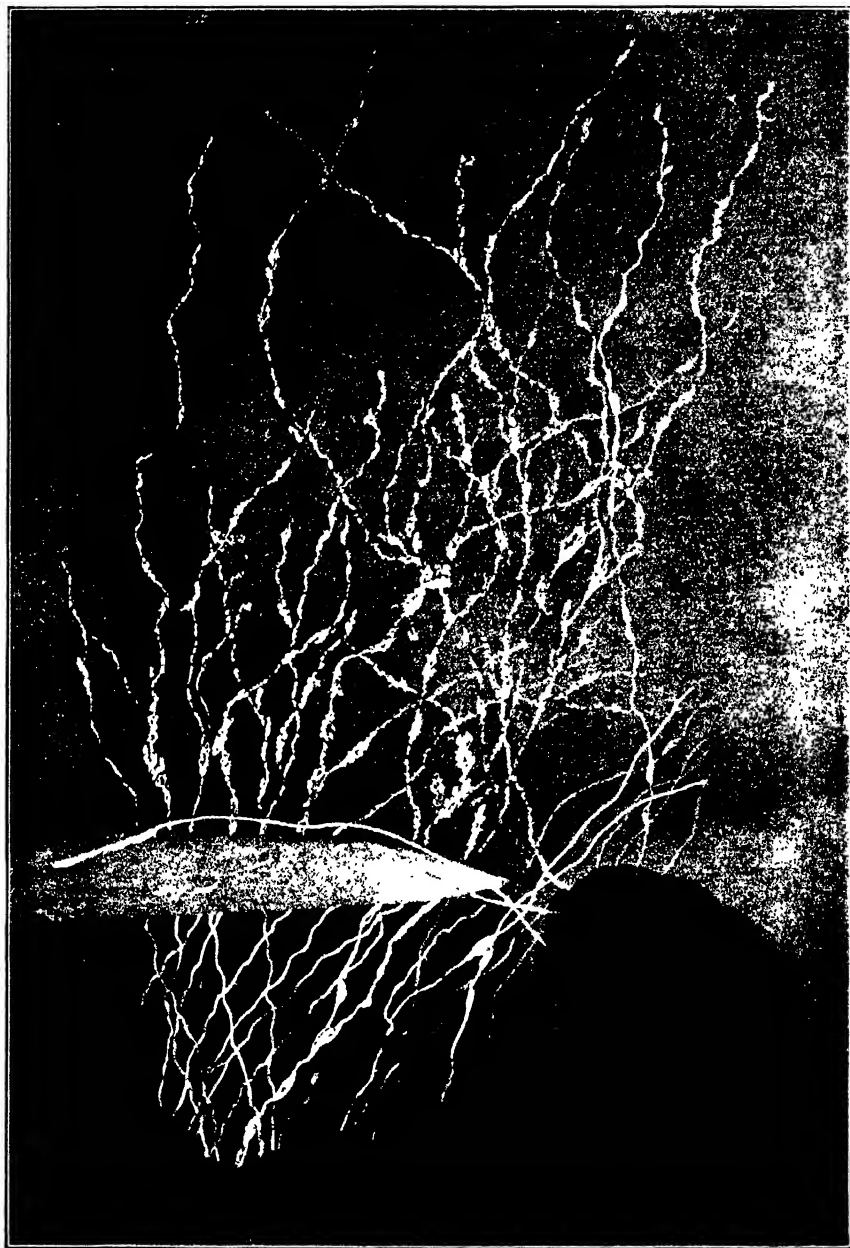


FIG. 14.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders *in Vitro*.)





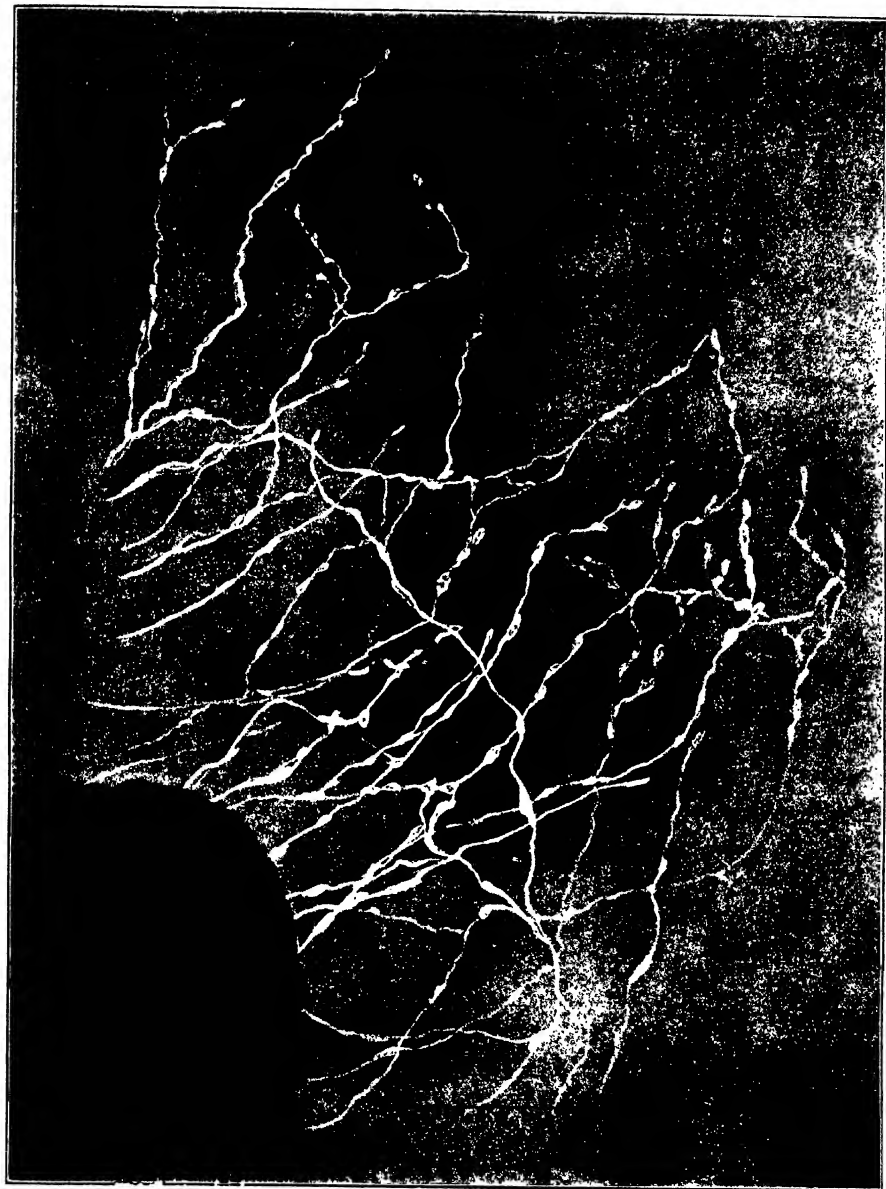


FIG. 15.

(Ingebrigtsen: Degeneration and Regeneration of Axis Cylinders *in Vitro*.)



PLATE 32.

FIG. 5. Connective tissue cells from a four day old culture of spinal ganglion of a cat six weeks old. Stained with hematoxylin.

PLATE 33.

FIG. 6. Culture of brain cortex three days old from a chick embryo eighteen days old.

FIG. 7. Culture of spinal medulla three days old from a chick embryo fourteen days old.

PLATE 34.

FIG. 8. Culture of spinal ganglion four days old from a rabbit seven months old.

PLATE 35.

FIG. 9. Culture of spinal ganglion two days old from a rabbit seven months old.

FIG. 10. Culture of brain cortex twenty-four hours old from a dog three weeks old.

PLATE 36.

FIG. 11. Culture of cerebellar cortex three days old from a dog three weeks old. Figures 12 to 15 are made from this culture in different stages.

PLATE 37.

FIG. 12. Culture of cerebellar cortex three days old from a dog three weeks old immediately after the cutting of the fibres. The gap is visible.

PLATE 38.

FIG. 13. Culture of cerebellar cortex four days old twenty hours after the cutting of the fibers which are slightly degenerated. New axis cylinders are seen in the gap.

PLATE 39.

FIG. 14. Culture of cerebellar cortex five days old forty-four hours after the cutting of the fibers which are still more degenerated. The new fibers in the gap have grown a little longer; one of them is slightly degenerated.

PLATE 40.

FIG. 15. Culture of cerebellar cortex five days old from a place just beyond the cut fibers. These axis cylinders are not degenerated.

## THE PERMANENT LIFE OF CONNECTIVE TISSUE OUTSIDE OF THE ORGANISM.\*

By ALBERT H. EBELING.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

PLATES 53 AND 54.

In previous articles<sup>1</sup> it has been demonstrated that connective tissue cells grow actively outside of the organism for more than four months and that a fragment of heart still pulsates after more than 100 days of life *in vitro*. The purpose of this article is to describe the continuation of these experiments.

The cultures of connective tissue on which Dr. Carrel made his observations were derived from sixteen small fragments of heart and blood vessels, extirpated on January 17, 1912, from chick embryos, seven to eighteen days old. These sixteen cultures did not grow actively. Several accidents occurred, and in March, 1912, only five cultures survived. After some modifications in the technique growth became more active. The mass of the new tissues increased rapidly. The tissues were divided and subdivided. In May, 1912, twenty-five to thirty cultures, derived from the few surviving cultures, were growing actively. The size of the piece of tissue contained in each culture increased very much during the latter part of May, 1912. Large necrotic areas appeared in the center of the tissues, and the growth now became less active.

I took charge of these cultures on June 1, 1912. They were washed in Ringer solution and transferred to a fresh medium every second, third, or fourth day, according to the technique described by Dr. Carrel.<sup>2</sup> The growth was slow and several bacterial infections occurred, so that on July 1 only five cultures survived. These, however, were growing actively. As the fragments were very small it was not possible to divide the tissues. In the beginning of September they were in good condition (figures 1 and 2). Technical accidents caused the loss of several cultures, and on September 25 only one culture survived. This culture arose from a fragment of

\* Received for publication, November 26, 1912.

<sup>1</sup> Carrel, A., *Jour. Exper. Med.*, 1912, xv, 516; Pozzi, F., *Bull. de l'Acad. de méd.*, 1912, lxvii, 475.

<sup>2</sup> Carrel, A., *loc. cit.*

connective tissue derived indirectly from the fragment of heart which still pulsated after 104 days of life *in vitro*. It was very small but grew actively. By repeated washings and passages the rate of growth was activated. On October 23 the piece of tissue began to increase rapidly and was divided in two parts. These cultures grew well and quickly and were divided and subdivided again and again. On November 17 the mass of tissue had increased, and twelve large cultures were finally obtained from the culture left in October. In January, 1913, there were thirty cultures.

The tissues contained in these cultures originated, as stated, indirectly from a piece of heart extirpated on January 17, 1912. Their growth was interrupted 128 or 129 times by a short washing in Ringer solution and by passage into a fresh medium. The history of these tissues is briefly described in table I. In December, 1912, the appearance of the cultures was about the same as of those which have been described in another article.<sup>3</sup> From a central mass of coagulated plasma and cells, new tissue, composed of elongated and ramified cells densely matted, grew out concentrically (figures 3 and 4).

The rate of growth following a passage can be seen by comparison with the width of the concentric ring of connective tissue surrounding the original central piece transferred. In many cases the rate of growth was modified by the osmotic tension, the composition of the medium, the manner in which the piece of tissue was cut out for the transfer, and the frequency of the passages. Nevertheless, growth during the eleventh month of the life of these tissues was very much more active than the growth of a fragment of heart of an eight day old chick embryo, and the mass of the tissues increased rapidly.

The experiments show that connective tissue can be kept in a condition of active growth outside of the organism for more than eleven months,<sup>4</sup> that its mass increases considerably, and its power of proliferation, after such a long period, is more active than at the beginning of its life *in vitro*.

<sup>3</sup> Carrel, A., *loc. cit.*

<sup>4</sup> On February 3, 1913, the tissues derived from this culture had undergone their 138th passage and were in a condition of active growth after more than one year of life *in vitro*.

TABLE I.

Passage.	Date (1912).	Treatment of culture.	Observations.
Experiment 725	Jan. 17	Culture of heart tissue from 7 day old chick embryo cultivated in chick plasma.	Jan. 19, pulsations had disappeared.
1	Jan. 19	Washed in Ringer solution for 5 minutes and cultivated in hypotonic chick plasma. <sup>a</sup>	Jan. 19, after transfer the heart fragment pulsated 48 times per minute, and connective tissue cells began to grow. Jan. 25, pulsations had disappeared.
2	Jan. 25	Washed in Ringer solution for 5 minutes and cultivated in hypotonic plasma.	Slow growth.
3	Jan. 30	Washed in Ringer solution for 5 minutes and cultivated in hypotonic plasma.	Slow growth.
4	Feb. 1	Washed in Ringer solution for 5 minutes and cultivated in hypotonic plasma, 2 drops, plus extract, 1 drop. <sup>a</sup>	Feb. 2, active growth.
5	Feb. 5	Washed in Ringer solution for 2 minutes and cultivated in hypotonic plasma.	Growing.
6	Feb. 7	Washed in Ringer solution for 2 minutes and cultivated in hypotonic plasma.	Growing.
7	Feb. 9	Washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma.	Growing.
8	Feb. 12	Washed in Ringer solution for 2 minutes and cultivated in hypotonic plasma.	Growing.
9	Feb. 13	Washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma.	Growing.
10	Feb. 16	Washed in Ringer solution for 2 minutes and cultivated in hypotonic plasma.	Good growth.
11	Feb. 19	Washed in Ringer solution for 4 minutes and cultivated in hypotonic plasma.	Good growth.
12	Feb. 23	Washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma.	Growing.

<sup>a</sup> Hypotonic plasma consisted of 3 parts of plasma and 2 parts of distilled water.

<sup>a</sup> The extract was prepared from seven day old chick embryo plus 3 drops of Ringer solution.

Passage.	Date (1912).	Treatment of culture.	Observations.
13	Feb. 27	Washed in Ringer solution for 2 minutes and cultivated in hypotonic plasma.	Good growth.
14	Feb. 28	Washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma.	Good growth.
15	Feb. 29	Washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma.	Pulsations reappeared. March 1, 9 A. M., 88 pulsations at 40° C.
16	March 4	Washed in Ringer solution for 2 minutes and cultivated in hypotonic plasma.	March 5, 9 A. M., 112 pulsations at 39° C.
17	March 8	Washed in Ringer solution for 2 minutes and cultivated in hypotonic plasma.	Ameboid and elongated tissue cells growing actively. March 8, 2 P. M., 48 pulsations at 40° C. March 13, 60 pulsations at 32° C. before changing culture. Fair growth of new cells.
18	March 13	Washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma plus a small drop of extract.	Good growth.
19	March 16	Washed in Ringer solution for 2 minutes and cultivated in hypotonic plasma, 2 drops, plus fresh extract, 1 drop.	12 pulsations at 39° C. before changing culture. (Irregular pulsations, intermittent, series of 4 or 5 pulsations, then long pause.)
20	March 19	Washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma, 2 drops, plus fresh embryonic extract, 1 drop.	Pulsations slow and irregular.
21	March 22	Washed in Ringer solution for 2 minutes and cultivated in hypotonic plasma, 2 drops, plus fresh embryonic extract, 1 drop.	124 pulsations at 40° C., again regular and vigorous. 24 to 28 pulsations.
22	March 26	Washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma, 2 drops, plus fresh embryonic extract, 1 drop.	44 pulsations, vigorous. Large growth of ameboid cells.
23	March 29	Washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma, 2 drops, plus fresh embryonic extract, 1 drop.	52 pulsations. Large increase in growth of cells.



Passage.	Date (1912).	Treatment of culture.	Observations.
24	April 1	Washed in Ringer solution for 4 minutes and cultivated in hypotonic plasma, 2 drops, plus fresh embryonic extract, 1 drop.	Folding in of piece in culture. April 2, no pulsations. The fragment of heart is imbedded in the middle of a very thick layer of cells and coagulated plasma.
25	April 2	Washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma, 2 drops, plus extract, 1 drop.	56 pulsations, vigorous after passage. Good growth.
Experiment 1269-2. 26	April 5	Culture 1239 was divided and washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma, 3 drops, plus fresh embryonic extract, 1 drop.	April 7, large growth, 52 pulsations at 39° C.; elongated and ameboid cells. April 8, large growth. The area covered by cells is equal to 70 times the area of fragment of heart tissue.
27	April 8	Washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma, 2 drops, plus fresh embryonic extract, 1 drop. <sup>7</sup>	April 11, very large growth.
28	April 11	Washed in Ringer solution for 2 minutes and cultivated in hypotonic plasma, 2 drops, plus fresh embryonic extract, 1 drop.	Large growth of fusiform and ameboid cells. April 13, very large growth.
29	April 15	Washed in Ringer solution for 2 minutes and cultivated in same medium.	April 17, large growth of cells.
30	April 18	Washed in Ringer solution for 2 minutes and cultivated in same medium.	April 19, network of elongated cells growing out from central piece of tissue.
31	April 22	Washed in Ringer solution for 4 minutes and cultivated in same medium.	Good growth.
32	April 25	Washed in Ringer solution for 2 minutes and cultivated in same medium.	April 28, good growth.
33	April 27	Washed in Ringer solution for 2 minutes and cultivated in same medium.	April 28, good growth.
34	April 30	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Good growth.

<sup>7</sup> Culture 1269-1 contained a pulsating fragment of heart, culture 1269-2 contained connective tissue cells which had grown out from the heart fragment in forty-eight hours.

Passage.	Date (1912).	Treatment of culture.	Observations.
35	May 3	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Good growth.
36	May 7	Washed in Ringer solution for 3 minutes and cultivated in same medium. (Culture was not extirpated from cover-glass, fresh medium was added.)	May 8, rapid growth.
37	May 10	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Good growth.
38	May 13	Washed in Ringer solution for 3 minutes and cultivated in same medium.	Growing.
39	May 16	Washed in Ringer solution for 3 minutes and cultivated in same medium.	Growing slowly.
40	May 18	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Large growth of ameboid cells.
41	May 20	Washed in Ringer solution for 2 minutes and cultivated in same medium.	May 21, good growth.
42	May 23	Washed in Ringer solution for 3 minutes and cultivated in same medium.	Growing slowly.
43	May 25	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Good growth.
44	May 28	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Growing actively; elongated and ameboid cells.
45	May 31	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Good growth; elongated and ameboid cells.
46	June 3	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Good growth.
47	June 6	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Good growth; elongated and ameboid cells.

Passage.	Date (1912).	Treatment of culture.	Observations.
48	June 8	Washed in Ringer solution for 2 minutes and cultivated in same medium.	A few cells growing.
49	June 11	Washed in Ringer solution for 2 minutes and cultivated in same medium.	A few cells growing.
50	June 14	Washed in Ringer solution for 2 minutes and cultivated in same medium.	A few cells growing.
51	June 17	Washed in Ringer solution for 2 minutes and cultivated in same medium.	A few cells growing.
52	June 21	Washed in Ringer solution for 2 minutes and cultivated in same medium.	A few cells growing.
53	June 24	Washed in Ringer solution for 2 minutes and cultivated in same medium.	A few cells growing.
54	June 27	Washed in Ringer solution for 3 minutes and cultivated in same medium.	Slight increase in growth.
55	July 1	Washed in Ringer solution for 3 minutes and cultivated in same medium. Ameboid cells growing.	Ameboid cells growing.
56	July 3	Washed in Ringer solution for 3 minutes and cultivated in same medium.	July 5, no growth.
57	July 6	Washed in Ringer solution for 3 minutes and cultivated in same medium.	July 8, growing well; ameboid cells predominate.
58	July 10	Washed in Ringer solution for 3 minutes and cultivated in same medium.	No growth.
59	July 12	Washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma, 2 drops, plus extract, 1 drop.	July 15, growing poorly; more ameboid cells present than elongated cells.
60	July 15	Washed in Ringer solution for 2 minutes and cultivated in normal plasma and extract.	July 17, active growth.
61	July 18	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Active growth of elongated cells; dense portion very small; cells growing out.

Passage.	Date (1912).	Treatment of culture.	Observations.
62	July 22	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Elongated and ameboid cells. Five old cultures still growing.
63	July 25	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Vigorous growth.
64	July 29	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Growing vigorously.
65	Aug. 1	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Culture 2001 divided into 3 parts; each piece retained a portion of the old mass.
66	Aug. 3	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Growing poorly.
67	Aug. 6	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Growing poorly.
68	Aug. 9	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Growing slowly. Dense central mass; active cells growing out.
69	Aug. 12	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Aug. 12. The older portion of the piece was discarded, the outer zone of active cells being transferred. Aug. 14, very luxuriant growth.
70	Aug. 15	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Aug. 16, growing fairly well.
71	Aug. 19	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Aug. 20, growing slowly.
72	Aug. 21	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Growing well; elongated cells.
73	Aug. 23	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Luxuriant growth; ameboid cells; network of elongated cells.
74	Aug. 26	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Aug. 26, growing well; infection of various cultures.
75	Aug. 28	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Aug. 31, extensive growth.

Passage.	Date (1912).	Treatment of culture.	Observations.
76	Sept. 3	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Photograph taken Sept. 3, 9.30 A. M., before passage and six days after change into fresh medium. Growing well.
77	Sept. 5	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Sept. 5, cover-glass broken; no infection. Sept. 7, vigorous growth. Sept. 9, growing well.
78	Sept. 9	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Growing fairly well.
79	Sept. 13	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Growing vigorously.
80	Sept. 16	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Growing vigorously.
81	Sept. 19	Washed in Ringer solution for 2 minutes and cultivated in same medium.	Growing vigorously. Sept. 21, growing vigorously.
82	Sept. 23	Washed in Ringer solution for 4 minutes and cultivated in same medium.	Good growth.
83	Sept. 25	Washed in Ringer solution for 3 minutes and cultivated in same medium.	Growing.
84	Sept. 28	Washed in Ringer solution for 3 minutes and cultivated in hypotonic plasma.	Growing.
85	Oct. 1	Washed in Ringer solution for 4 minutes and cultivated in plasma, 2 drops, plus extract, 1 drop.	Good growth.
86	Oct. 4	Washed in Ringer solution for 3 minutes and cultivated in plasma, 2 drops, plus extract, 1 drop.	Good growth.
87	Oct. 7	Washed in Ringer solution for 1 minute and cultivated in plasma, 2 drops, plus extract, 1 drop.	Good growth.
88	Oct. 10	Washed in Ringer solution for 1 minute and cultivated in plasma (1 part plasma, 3 parts water), 2 drops, plus extract, 1 drop.	Good growth.

Passage.	Date (1912).	Treatment of culture.	Observations.
89	Oct. 12	Washed in Ringer solution for 1 minute and cultivated in same medium.	Excellent growth. Oct. 13-15, excellent growth.
90	Oct. 16	Washed in Ringer solution for 1 minute and cultivated in same medium.	Oct. 17, excellent growth of elongated cells.
91	Oct. 19	Washed in Ringer solution for 1 minute and cultivated in same medium.	Oct. 21, very good growth.
92	Oct. 23	Washed in Ringer solution for 1 minute and cultivated in same medium.	Oct. 24, 7 A. M., large growth of scattered cells. Oct. 25, excellent growth. Oct. 25, 9 P. M., large growth.
93	Oct. 26	Washed in Ringer solution for 30 seconds and cultivated in same medium.	Oct. 26, 11 P. M., good growth.
94	Oct. 29	Washed in Ringer solution for 50 seconds and cultivated in same medium.	Excellent growth.
95	Nov. 1	Culture 2611 divided into 2 parts, washed in Ringer solution for 30 seconds, and cultivated in same medium.	Nov. 1, mass had increased to such an extent that culture was divided into 2 parts. Nov. 2, rapid growth. Nov. 4, great increase in mass and density.
96	Nov. 2	Washed in Ringer solution for 35 seconds and cultivated in same medium.	Nov. 2, 6 P. M., excellent growth 9 hours after changing into new medium. Nov. 4, excellent growth, very dense.
97	Nov. 5	Culture 2647 divided into 2 parts, washed in Ringer solution for 35 seconds, and cultivated in same medium.	Nov. 5, cultures were divided again, making 4. Excellent growth. Nov. 6, infection.
98	Nov. 7	Culture 2662 divided into 2 parts; infected area removed. Washed in Ringer solution for 35 seconds and cultivated in same medium.	Nov. 7, cultures divided again, making 8. Excellent growth.
99	Nov. 9	Culture put directly into new medium without washing.	Growing fairly well.
100	Nov. 12	Washed in Ringer solution for 25 seconds and cultivated in same medium.	Excellent growth.

Passage.	Date (1912).	Treatment of culture.	Observations.
101	Nov. 14	Culture 2734 divided into 2 parts, washed in Ringer solution for 30 seconds, and cultivated in same medium.	Nov. 14, cultures divided again, but cultivated on the same cover-glass.
102	Nov. 17	Culture 2765 divided into 2 parts, washed in Ringer solution for 40 seconds, and cultivated in same medium.	Nov. 17, cultures divided again, making 16 separate cultures. Excellent growth.
103	Nov. 19	Washed in Ringer solution for 1½ minutes and cultivated in same medium.	Excellent growth.
104	Nov. 21	Washed in Ringer solution for 30 seconds and cultivated in same medium.	Nov. 22, excellent growth.
105	Nov. 23	Washed in Ringer solution for 35 seconds and cultivated in same medium.	Nov. 24 and 25, excellent growth.
106	Nov. 25	Washed in Ringer solution for 38 seconds and cultivated in same medium.	Nov. 25, 2 P. M., 3 hours after change, many elongated cells growing out. Nov. 26, excellent growth. Photograph taken.
107	Nov. 27	Washed in Ringer solution for 30 seconds and cultivated in same medium.	Nov. 28 and 29, excellent growth, dense network of cells.
108	Nov. 30	Washed in Ringer solution for 30 seconds and cultivated in same medium.	Dec. 1 and 3, excellent growth.
109	Dec. 3	Washed in Ringer solution for 40 seconds and cultivated in same medium.	Dec. 4, excellent growth.
110	Dec. 4	Washed in Ringer solution for 30 seconds and cultivated in same medium.	Dec. 5, excellent growth.
111	Dec. 5	Culture partially divided, washed in Ringer solution for 30 seconds, and cultivated in same medium.	Dec. 5, 3.50 P. M., excellent growth 5 hours after transfer.
112	Dec. 7	Culture partially divided, washed in Ringer solution for 35 seconds, and cultivated in same medium.	Dec. 8, excellent growth, quite dense.
113	Dec. 10	Washed in Ringer solution for 1 minute and cultivated in same medium.	Dec. 11, excellent growth.

Passage.	Date (1912).	Treatment of culture.	Observations.
114	Dec. 12	Washed in Ringer solution for 30 seconds and cultivated in normal plasma, 2 drops, plus extract (24 hours old and kept in cold storage), 1 drop.	Dec. 13, excellent growth.
115	Dec. 14	Washed in Ringer solution for 35 seconds and cultivated in normal plasma, 2 drops, plus extract (24 hours old), 1 drop.	Dec. 14, 1 P. M., new cells growing out 3 hours after transfer.
116	Dec. 16	Culture divided into 2 parts, washed in Ringer solution for 30 seconds, and cultivated in same medium.	Dec. 19, excellent growth.
117	Dec. 17	Culture divided into 2 parts. Nos. 1 and 2 washed in Ringer solution for 30 seconds. No. 1 cultivated in normal plasma and old extract (prepared Dec. 15).	Dec. 17, 6 P. M., excellent growth; a crown of new cells growing rapidly. Dec. 18, A. M., excellent growth, quite dense.
118	Dec. 19	Washed in Ringer solution for 30 seconds and cultivated in same medium.	Dec. 19, 6 P. M., excellent growth. Dec. 20, A. M., excellent growth, fairly dense.
119	Dec. 21	Culture divided, washed in Ringer solution for 40 seconds, and cultivated in same medium.	Dec. 22, excellent growth, quite extensive.
120	Dec. 24	Culture divided, washed in Ringer solution for 35 seconds, and cultivated in plasma, 2 drops, plus fresh extract, 1 drop.	Dec. 26, excellent growth; area increased about four times.
121	Dec. 27	Culture divided, washed in Ringer solution for 30 seconds, and cultivated in same medium.	Dec. 28, poor growth. Dec. 29, growing slowly. Dec. 30, good growth; many scattered cells grow in a medium due to extract.
122	Dec. 30	Culture divided, washed in isotonic <sup>a</sup> solution for 30 seconds and cultivated in plasma, 2 drops, plus old extract (prepared Dec. 21 and kept in cold storage), 1 drop.	Jan. 2, excellent growth; increase in mass about 4 times original size.

<sup>a</sup> The isotonic solution consisted of sodium chloride, 8.5 gm.; sodium bicarbonate, 0.3 gm.; calcium chloride, 0.25 gm.; potassium chloride, 0.1 gm.; and distilled water to make 1,000 gm.



Passage.	Date (1913).	Treatment of culture.	Observations.
123	Jan. 2	Culture divided into 2 parts, washed in isotonic solution for 35 seconds, and cultivated in plasma, 2 drops, plus old extract (prepared Dec. 27), 1 drop.	Jan. 2, before passage. Excellent growth; increase in mass about 4 times.
124	Jan. 4	Washed in isotonic solution for 30 seconds and cultivated in plasma, 2 drops, plus old extract (prepared Jan. 3), 1 drop.	Jan. 5, good growth.
125	Jan. 6	Washed in isotonic solution for 30 seconds and cultivated in plasma, 2 drops, plus brain extract (prepared from brain of adult chicken, Dec. 19), 1 drop.	Jan. 7, good growth.
126	Jan. 9	Washed in Ringer solution for 30 seconds and cultivated in plasma, 2 drops, plus fresh extract, 1 drop.	Jan. 10, growing fairly well.
127	Jan. 11	Washed in Ringer solution for 35 seconds and cultivated in same medium.	Jan. 12, growing.
128	Jan. 14	Washed in isotonic solution for 1 minute and cultivated in same medium.	Jan. 15, growing fairly well.
129	Jan. 15	Washed in Ringer solution for 30 seconds and cultivated in same medium.	Jan. 15, 4.30 P. M., new cells growing forth. Jan. 16, 9 A. M., very good growth.

## EXPLANATION OF PLATES.

## PLATE 53.

FIG. 1. A culture of connective tissue derived from embryonic heart tissue (chick) which was extirpated on January 17, 1912. The photograph was taken on September 3, 1912, six days after the seventy-fifth passage.

FIG. 2. The same as figure 1; high magnification.

## PLATE 54.

FIG. 3. A culture of connective tissue derived from the same culture as figure 1. The photograph was taken on November 25, 1912, forty-eight hours after the 105th passage. The photograph shows an opaque area composed of cells and old plasma which is surrounded by a clearer area of the tissue formed in forty-eight hours.

FIG. 4. The same as figure 3; higher magnification. The photograph shows part of the area of tissue formed in forty-eight hours.



FIG. 1.



FIG. 2.

(Ebeling : Life of Connective Tissue.)





FIG. 4.

(Ebeling : Life of Connective Tissue.)



## THE COMPARATIVE EFFICIENCY OF WEAK AND STRONG BASES IN ARTIFICIAL PARTHENOGENESIS.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

In 1905 the writer found that it is possible to induce artificial parthenogenesis (membrane formation) in the sea urchin by weak acids, such as the monobasic fatty acids or  $\text{CO}_2$ , but not at all or only unsatisfactorily by the strong acids, such as  $\text{HCl}$ ,  $\text{H}_2\text{SO}_4$ , oxalic acid, and others. He suggested that this paradoxical behavior was due to the fact that only those acids which diffuse easily into the egg were able to cause membrane formation.<sup>1</sup> This assumption was supported by the observation that there existed an analogy between the relative physiological efficiency of various organic acids and their corresponding alcohols.

This paper intends to show that the weak base  $\text{NH}_4\text{OH}$  is much more efficient in the production of artificial parthenogenesis than the strong bases  $\text{NaOH}$ ,  $\text{KOH}$ , and tetraaethylammoniumhydroxide. The writer found in 1907 that it is possible to substitute bases for acids in the process of artificial parthenogenesis with this difference, that the eggs had to be exposed to the alkaline solution for a considerably longer period than to the acid solution in order to cause them to develop.<sup>2</sup> The eggs of *Strongylocentrotus* could be caused to develop by putting them for nearly three hours into a mixture of 50 cc.  $\text{m}/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) + 0.5 or 1.0 cc.  $\text{N}/10$   $\text{NaOH}$ . When such eggs were then transferred for from thirty-five to forty minutes to a hypertonic solution (50 cc. sea water + 8 cc.  $2\frac{1}{2}$  m

<sup>1</sup>Loeb, University of California Publications, vol. 2, p. 113, 1905. Also in "Die chemische Entwicklungserregung des tierischen Eies," p. 100, Berlin, 1909.

<sup>2</sup>Loeb, Ueber die allgemeinen Methoden der künstlichen Parthenogenese, Pflüger's Archiv, Bd. 118, p. 572, 1907.

$\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) a number of them formed membranes and the eggs developed afterward into swimming larvae.

Recently O. Warburg<sup>3</sup> showed that  $\text{NH}_4\text{OH}$  diffuses rapidly into the sea urchin egg while  $\text{NaOH}$  does not, and this observation was confirmed and enlarged by Harvey.<sup>4</sup> This suggested the possibility that, as in the case of acids, weak bases might be found to be more effective in producing artificial parthenogenesis than strong bases.

#### I. METHOD.

In order to obtain comparable results the bases had to be added to a neutral solution instead of to sea water. An  $m/2$  solution of  $(\text{NaCl} + \text{CaCl}_2 + \text{KCl})$  in the usual proportion was used<sup>5</sup> for this purpose. Before the eggs were put into this solution they were freed from sea water by repeated washing in a solution of the same constitution and concentration. From the alkaline solution the eggs were transferred directly into the neutral hypertonic solution. The latter consisted of 50 cc.  $m/2$   $(\text{NaCl} + \text{CaCl}_2 + \text{KCl}) + 8$  cc.  $2\frac{1}{2}$   $m$  of the same mixture. From the hypertonic solution the eggs were transferred to normal sea water. They often showed a tendency to stick to the glass. This was overcome by preventing them from settling for about five minutes through gentle agitation.

#### 2. COMPARISON OF THE EFFICIENCY OF $\text{NH}_4\text{OH}$ AND $\text{KOH}$ .

To 50 cc.  $m/2$   $(\text{NaCl} + \text{KCl} + \text{CaCl}_2)$ , 0.3 cc.  $N/10$   $\text{NH}_4\text{OH}$ , and 0.3 cc.  $N/10$   $\text{KOH}$  were added respectively. Unfertilized eggs of *Arbacia* were left in these solutions for six, twelve, twenty-four, forty-two, and sixty-one minutes. Then they were transferred for fifteen minutes into the neutral hypertonic solution, namely, 50 cc.  $m/2$   $(\text{NaCl} + \text{KCl} + \text{CaCl}_2) + 8$  cc.  $2\frac{1}{2}$   $m$   $(\text{NaCl} + \text{KCl} + \text{CaCl}_2)$ . From the hypertonic solution they were transferred into normal sea water. The temperature varied but little from  $22^\circ \text{C}$ . The results of the experiment follow.

<sup>3</sup> O. Warburg, *Zeitsch. f. physiolog. Chem.*, Bd. 66, p. 305, 1910.

<sup>4</sup> Harvey, *Jour. Exper. Zool.*, vol. 10, p. 507, 1911.

<sup>5</sup> This proportion is as follows: 100 cc.  $m/2$   $\text{NaCl} + 2.2$  cc.  $m/2$   $\text{KCl} + 1.5$  cc.  $m/2$   $\text{CaCl}_2$ .

*a. Eggs Six Minutes in the Alkaline Solution.*—Some of the eggs which had been in  $\text{NH}_4\text{OH}$  for six minutes developed as far as the two or even the four cell stage, but no further. The blastomeres of the segmented eggs fell apart. No larvae were formed and the majority of the eggs remained unaltered. None of the eggs that had been in KOH for six minutes segmented; all remained unaltered. The eggs which did not segment had no membranes.

*b. Eggs Twelve Minutes in Alkali.*—A large percentage of the eggs that had been twelve minutes in the  $\text{NH}_4\text{OH}$  formed membranes and segmented, and a few of these developed into larvae. The eggs which had not formed membranes remained unsegmented and intact.

The eggs which had been for twelve minutes in KOH formed no membranes and did not segment or develop into larvae. A few showed amoeboid changes preceding a possible cell division. Practically all the eggs were intact on the following day.

*c. Eggs Twenty-Four Minutes in Alkali.*—The eggs that had been in  $\text{NH}_4\text{OH}$  for twenty-four minutes practically all formed membranes, segmented normally to a large extent, and formed larvae. Many of the latter reached the pluteus stage, and swam at the surface of the dish.

Of the eggs that had been twenty-four minutes in KOH about 10 per cent formed membranes and began to segment, but did not go beyond the first stages of segmentation. Ninety per cent of the eggs formed no membranes, did not segment, and remained unaltered. We shall see later that such eggs, upon the addition of sperm, will develop into normal larvae, thus indicating that the treatment with KOH did not affect them.

*d. Eggs Forty-Two Minutes in Alkali.*—About 90 per cent of the eggs that had been in  $\text{NH}_4\text{OH}$  for forty-two minutes formed membranes, segmented and developed into swimming larvae. Not so many reached the pluteus stage as in the previous lot.

Only a small percentage of the eggs that had been in KOH for forty-two minutes formed membranes and segmented, and only a few of these developed into swimming larvae.

*e. Eggs Sixty Minutes in Alkali.*—The eggs that had been in  $\text{NH}_4\text{OH}$  for sixty minutes formed membranes. Some began to seg-



ment but the majority disintegrated without reaching the larval stage.

A considerable percentage of the eggs treated with KOH for sixty minutes began to segment, but most of them disintegrated before they reached the blastula stage. The rest of the eggs remained intact.

We may summarize the result of this experiment by saying *that practically all the eggs that had been treated with a 3/5000 N solution of  $\text{NH}_4\text{OH}$  for twenty-four minutes and were then put into a neutral hypertonic solution for fifteen minutes developed into larvae, this development being normal in a large number of cases. The eggs, however, that were treated with a 3/5000 N solution of KOH for twenty-four minutes and then put into a neutral hypertonic solution for fifteen minutes remained practically unaltered.*

### 3. COMPARISON OF THE EFFICIENCY OF $\text{NH}_4\text{OH}$ , $\text{NaOH}$ AND TETRAETHYLAMMONIUMHYDROXIDE.

To three solutions of 50 cc.  $\text{m}/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) were added 0.3 cc.  $\text{N}/10$   $\text{NH}_4\text{OH}$ , 0.3 cc.  $\text{N}/10$   $\text{NaOH}$ , and 0.3 cc.  $\text{N}/10$  tetraethylammoniumhydroxide respectively. Unfertilized eggs of *Arbacia* were put into these solutions for twenty-six minutes and were then transferred directly into a neutral hypertonic solution, namely 50 cc.  $\text{m}/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) + 8 cc.  $2\frac{1}{2}$   $\text{m}$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ). They remained here for fifteen minutes and were then transferred into normal sea water.

Practically all the eggs that had been in 0.3 cc.  $\text{NH}_4\text{OH}$  for twenty-six minutes developed into larvae (about as quickly as the larvae from fertilized eggs); the eggs that had been in 0.3 cc.  $\text{NaOH}$ , or in 0.3 cc. tetraethylammoniumhydroxide for twenty-six minutes remained practically intact. Only a few eggs segmented, and only after a long search was it possible to find a swimming larva on the following day.

*These experiments, which were repeated a number of times, indicate that the weak base  $\text{NH}_4\text{OH}$  is much more efficient in the causation of artificial parthenogenesis than the strong bases  $\text{NaOH}$ ,  $\text{KOH}$ , and tetraethylammoniumhydroxide.*

#### 4. ACTION OF ALKALIES ALONE, WITHOUT THE ACTION OF HYPERTONIC SOLUTION.

0.3 cc.  $N/10$   $NH_4OH$ , 0.3 cc.  $N/10$   $NaOH$ , and 0.3 cc.  $N/10$  tetraethylammoniumhydroxide were added to 50 cc.  $m/2$  ( $NaCl + KCl + CaCl_2$ ) respectively. Unfertilized eggs of *Arbacia* were put into these three solutions for forty-two minutes and then transferred to normal sea water. All of the eggs that had been in the solution containing the  $NH_4OH$  segmented in a rather amoeboid way into two or four cells, after which the cells fell apart and disintegrated. All of the eggs that had been in 50 cc.  $m/2$  ( $NaCl + KCl + CaCl_2$ ) + 0.3 cc.  $N/10$   $NaOH$  for forty-two minutes remained practically intact and the same was true for the eggs that had been in the tetraethylammoniumhydroxide for forty-two minutes. In order to make sure that they did not only appear normal but were normal, sperm was added to these eggs the next morning. All the eggs that had been in  $NaOH$ , and in tetraethylammoniumhydroxide, segmented normally and developed into normal embryos.

In this experiment part of the eggs were submitted for fifteen minutes to the action of the neutral hypertonic solution after they had been treated with alkali. The eggs that had been in  $NH_4OH$  developed into larvae, the others did not. It is obvious that the changes leading to parthenogenetic development are brought about considerably more rapidly by  $NH_4OH$  than by the strong bases.

All this is in complete analogy with the action of acids in artificial parthenogenesis. Only the acid or alkali which enters the egg can act, and since  $NH_4OH$  enters much more rapidly than the strong bases, the weak base  $NH_4OH$  is more efficient than the strong bases.

#### 5. OXIDATION AND ACTION OF ALKALI IN ARTIFICIAL PARTHENOGENESIS.

In former papers the writer had shown that the parthenogenetic as well as the destructive action of  $KOH$  and  $NaOH$  upon cells can be retarded or suppressed through the removal of oxygen or

the addition of a few drops of KCN.<sup>6</sup> It was our intention to find out whether the action of  $\text{NH}_4\text{OH}$  in artificial parthenogenesis could also be suppressed by KCN. This is indeed the case. Two solutions of 50 cc.  $\text{m}/2$  ( $\text{NaCl} + \text{CaCl}_2 + \text{KCl}$ ) + 0.3 cc.  $\text{N}/10$   $\text{NH}_4\text{OH}$  were prepared. To one of these were added five drops of a 0.1 per cent solution of KCN. Unfertilized eggs of *Arbacia* were put into these solutions for forty-five minutes and then transferred to sea water. The eggs which had been in the solution containing KCN remained absolutely intact and unaltered. The next morning sperm was added and all segmented regularly, developing into perfectly normal larvae. The eggs, however, which had been in the solution not containing KCN began to segment and in a few hours disintegrated completely.

If the eggs remain for a number of hours in a mixture of 50 cc.  $\text{m}/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) + 0.3 cc.  $\text{N}/10$   $\text{NH}_4\text{OH}$  + 5 drops of 0.1 per cent KCN they remain intact, but when put back into normal sea water they soon segment in an irregular way and disintegrate. This is in agreement with the well known fact that the amount of KCN added in this case only retards the oxidations but does not suppress them entirely.

These experiments throw a light upon the localization of oxidations in the cell. Warburg pointed out that the increase of the rate of oxidations in the egg by  $\text{NaOH}$  can only be ascribed to a surface action, since the  $\text{NaOH}$  does not noticeably diffuse into the egg. Wasteneys and I found that the weak base  $\text{NH}_4\text{OH}$  accelerates the rate of oxidations about one half as much as the strong base  $\text{NaOH}$ .<sup>7</sup> The fact that  $\text{NH}_4\text{OH}$  raises the rate of oxidations much more than should be expected from its low degree of dissociation becomes intelligible if we assume that the  $\text{NH}_4\text{OH}$  which diffuses into the egg influences the rate of oxidations more strongly than the alkali which acts merely on the surface of the egg. On this assumption the external surface of the egg is neither the only nor perhaps the main seat of oxidation, but oxidations occur also in the interior of the egg.

<sup>6</sup> Pflüger's Archiv, Bd. 118, p. 30, 1907, and Die chemische Entwicklungserregung des tierischen Eies, p. 118.

<sup>7</sup> Loeb and Wasteneys, Biochem. Zeitsch., Bd. 37, p. 410, 1911.

Another fact which would agree with such a view is the following: The egg of the Californian sea urchin, *Strongylocentrotus purpuratus*, cannot develop in an acid or in a neutral solution. It suffices, however, to add a small amount of neutral red to the salt solution to start the development. Neutral red is a much weaker base than  $\text{NH}_4\text{OH}$  and diffuses rapidly into the egg. It is not well conceivable that neutral red could accelerate the oxidations in the sea urchin egg except on the assumption that  $\text{NaOH}$  acts only or at least mainly on the surface, while neutral red acts in addition inside the egg. Since in this case the action of the alkali consists also in an acceleration of the rate of the oxidations this would also point towards the probability that the external surface is not the only seat of oxidations in the cell.

Finally, it should not be overlooked that the strong bases  $\text{NaOH}$  or  $\text{KOH}$  have also a small parthenogenetic effect. In view of the experiments mentioned in this paper this fact suggests the possibility that the strong bases diffuse into the egg to a slight extent, or at least into its cortical layer. It is probable that for this diffusion only the undissociated molecules of these bases are to be considered. The fact that the strong bases do not change the color of the neutral red contained in the egg does not contradict such an assumption, if we suppose that the base acts in the egg through salt formation with an acid constituent of the egg, e. g., an acid protein.

## 6. MEMBRANE FORMATION BY ALKALI.

In his former experiments on the parthenogenetic action of alkalis in *Strongylocentrotus* the writer pointed out that the eggs which are induced to develop under the influence of  $\text{KOH}$  form a membrane, but that this membrane formation takes place, as a rule, not in the alkaline solution but in the hypertonic solution. The egg of *Arbacia* does not form as distinct a membrane under the influence of alkali as does the egg of *Strongylocentrotus*. In *Arbacia*, as a rule, only a fine gelatinous layer is formed at the surface of the egg through the agencies of artificial parthenogenesis.

When the eggs of *Arbacia* are put for twenty-five minutes into a mixture of 50 cc.  $\text{m}/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) + 0.3 cc.  $\text{N}/10$   $\text{NH}_4\text{OH}$  and then transferred to a neutral hypertonic solution for

about fifteen minutes (at a temperature of about  $22^{\circ}$  C.), as a rule a large percentage, if not all the eggs, will develop into larvae and it will be found that the eggs which develop possess a membrane. The membrane in this case is not formed while the eggs are in the alkaline solution but while they are in the hypertonic solution. When the eggs are left in the alkaline solution much longer than twenty-five minutes membranes may be formed in the latter.

The following observation is of interest. Eggs were put for forty-five minutes into a mixture of 50 cc.  $m/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) + 0.3 cc.  $N/10$   $\text{NH}_4\text{OH}$  + 5 drops 0.1 per cent KCN. From here they were transferred for fifteen minutes into the neutral hypertonic solution and then into sea water. All these eggs formed membranes but perished by cytolysis, the pigment gathering as a rule in one or more spots of the egg. The reader will remember that such eggs when put directly from the alkaline solution with KCN into sea water (without undergoing a treatment with the neutral hypertonic solution) form no membrane, and remain intact, developing normally if later on fertilized by sperm.

Why then does the treatment of such eggs for fifteen minutes with the hypertonic solution kill them? The experiment seems to indicate that the  $\text{NH}_4\text{OH}$  has two effects, one of which consists in inducing the process of membrane formation. This process in this experiment was not inhibited through the presence of KCN in the  $\text{NH}_4\text{OH}$  solution. In former papers the writer has shown that the mere membrane formation leads to the death of the sea urchin egg unless the latter is put into the hypertonic solution for a sufficiently long time. If the eggs do not remain in the hypertonic solution a sufficient period of time after the artificial membrane formation they will perish. Such was the case in this experiment. In order to make this clear another set of experiments must be discussed.

#### 7. VARIATION OF THE TIME OF EXPOSURE TO THE HYPERTONIC SOLUTION.

In the experiments thus far mentioned the eggs of *Arbacia* were always exposed to the neutral hypertonic solution for fifteen minutes at a temperature of about  $22^{\circ}$  C. When the eggs had pre-

viously been treated for about twenty-five minutes with a mixture of 50 cc.  $m/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) + 0.3 cc.  $N/10$   $\text{NH}_4\text{OH}$  the exposure of fifteen minutes to a neutral hypertonic solution as a rule sufficed to cause all or many of the eggs to develop. If the eggs remain in the hypertonic solution for a longer period, they develop in a less regular way and perish, as a rule, at the time of the blastula formation, probably on account of irregular (multi-polar?) mitosis. A shorter exposure than fifteen minutes at this temperature is, as a rule, inadequate to protect the eggs from disintegrating during the first segmentation. As was to be expected from the author's former experiments, the optimal time of exposure of the eggs to the hypertonic solution varies, if the time of exposure to the alkaline solution varies.

Unfertilized eggs of *Arbacia* were put into a mixture of 50 cc.  $m/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) + 0.3 cc.  $N/10$   $\text{NH}_4\text{OH}$ . One part of the eggs was transferred to the neutral hypertonic solution after ten minutes, and the rest after thirty minutes. After different intervals some of the eggs were transferred to normal sea water. The result is indicated in the following table. Temperature  $23^\circ \text{C}$ .

(a) *Eggs in 0.3 cc.  $N/10$   $\text{NH}_4\text{OH}$  for ten minutes and subsequently in the neutral hypertonic solution for*

16 minutes: The eggs begin to segment, but disintegrate. No larvae formed.

24 minutes: *Many eggs develop into larvae which rise to the surface.*

32 minutes: Few eggs develop into larvae. The majority disintegrate.

45 minutes: All form membranes and begin to develop, but disintegrate.

60 minutes: Like the preceding lot.

(b) *Eggs in 0.3 cc.  $N/10$   $\text{NH}_4\text{OH}$  for thirty minutes and subsequently in the neutral hypertonic solution for*

10 minutes: About 10 per cent of the eggs form membranes and develop into perfect larvae.

15 minutes: *Practically all the eggs develop into swimming larvae, many of which are perfect.* Numerous larvae rise to the surface.

23 minutes: Very few eggs develop into larvae; the majority of the eggs undergo cytolysis.

45 minutes: All the eggs undergo cytolysis.

It is, therefore, obvious that the eggs that had been in 50 cc.  $m/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) + 0.3 cc.  $N/10$   $\text{NH}_4\text{OH}$  for ten minutes developed best when exposed to the hypertonic solution for twenty-

four minutes, while an exposure of sixteen minutes was too short and one of thirty-two minutes too long. The eggs that had been in the same alkaline solution three times as long (thirty minutes) developed best when put for fifteen minutes into the neutral hypertonic solution, while ten minutes were not quite sufficient and twenty-three minutes too long.

These observations throw a light on the experiment mentioned in the previous paragraph. In this experiment the eggs were put into an  $\text{NH}_4\text{OH}$  solution containing KCN for forty-five minutes. The latter retarded the oxidizing effect of the  $\text{NH}_4\text{OH}$  and therefore had the same effect as if the eggs had been put for a shorter period into an  $\text{NH}_4\text{OH}$  solution free from KCN. We have seen, however, that a shorter exposure of the eggs to a solution of  $\text{NH}_4\text{OH}$  requires a longer exposure than fifteen minutes to the hypertonic solution if we wish to cause the development of the eggs into larvae. The eggs did not develop in this experiment because the exposure of fifteen minutes to the hypertonic solution was in this case too short.

This idea was put to a test. The unfertilized eggs of *Arbacia* were put for forty-two minutes into a solution of 50 cc.  $\text{m}/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) + 0.3 cc.  $\text{N}/10$   $\text{NH}_4\text{OH}$  + 5 drops 0.1 per cent KCN. They were then transferred directly into the neutral hypertonic solution, 50 cc.  $\text{m}/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) + 8 cc.  $2\frac{1}{2}\text{m}$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ). Part of the eggs were transferred after fifteen, twenty-five and thirty-three minutes to normal sea water. Of the eggs that remained only fifteen minutes in the hypertonic solution all perished. About 50 per cent of those that had been in the hypertonic solution twenty-five minutes developed into larvae, and a still greater part of those that had been in the hypertonic solution for thirty-three minutes developed. A large number of these larvae rose to the surface. If the eggs had been in the  $\text{NH}_4\text{OH}$  solution without KCN an exposure of fifteen minutes to the hypertonic solution would have been sufficient.

It should also be remembered that the writer had shown long ago that the action of the hypertonic solution requires also the presence of free oxygen and is delayed through the addition of KCN. When the eggs are put into the alkaline solution containing KCN for forty minutes they will not at once lose all the KCN or HCN when put

into the hypertonic solution. This may be an additional reason for the necessity of keeping them longer than fifteen minutes in the hypertonic solution after a treatment with a KCN solution.

#### 8. EFFECT OF THE CONCENTRATION OF $\text{NH}_4\text{OH}$ .

In all the experiments mentioned thus far the concentration  $\text{NH}_4\text{OH}$  used was  $3/5000 \text{ N}$  ( $0.3 \text{ N}/10 \text{ NH}_4\text{OH}$ ) to 50 cc.  $\text{m}/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ), since this concentration was found to be very satisfactory for the production of good larvae. It was desirable to get an idea of the limits of the concentrations in which the  $\text{NH}_4\text{OH}$  can be used. To 50 cc.  $\text{m}/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) were added 0.05, 0.1, 0.2, 0.4, 0.8 cc.  $\text{N}/10 \text{ NH}_4\text{OH}$  and unfertilized eggs were put into these solutions. The eggs remained in the solutions forty minutes and were then transferred into the above mentioned neutral hypertonic solutions. They remained in the hypertonic solutions for fifteen minutes and were then transferred to normal sea water. The results are indicated in the following table:

##### *Amount of $\text{NH}_4\text{OH}$ used:*

0.05 cc. $\text{N}/10 \text{ NH}_4\text{OH}$	Two larvae found. Practically all the eggs unaltered and normal. No membranes.
0.10 cc. $\text{N}/10 \text{ NH}_4\text{OH}$	Very few larvae. About half of the eggs unaltered, the rest cytolized.
0.20 cc. $\text{N}/10 \text{ NH}_4\text{OH}$	Few larvae. Practically all the eggs cytolized.
0.40 cc. $\text{N}/10 \text{ NH}_4\text{OH}$	A large number of larvae, part of which rise to the surface.
0.80 cc. $\text{N}/10 \text{ NH}_4\text{OH}$	Very few larvae; the rest of the eggs practically all cytolized.

These results are easily intelligible in the light of the previously described experiments. The addition of 0.05 cc.  $\text{N}/10 \text{ NH}_4\text{OH}$  to 50 cc.  $\text{m}/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) does not affect the eggs in forty minutes, nor does an exposure to the hypertonic solution for fifteen minutes. Practically all of the eggs, therefore, remain normal. 0.10 cc.  $\text{N}/10 \text{ NH}_4\text{OH}$  is able to affect a number of eggs in forty minutes, but the exposure of fifteen minutes in the hypertonic solution is too short (see previous paragraph). Therefore, the affected eggs perish. They might have developed had they been exposed a little longer to the hypertonic solution. 0.04 cc.



N/10  $\text{NH}_4\text{OH}$  is satisfactory for an exposure of forty minutes to the alkaline solution and of fifteen minutes to the hypertonic treatment. This series, therefore, yields a good crop of larvae, although it is not the optimum. 0.8 cc. N/10  $\text{NH}_4\text{OH}$  is too high a concentration, for it injures the eggs and only a few survive.

#### 9. THE INDIVIDUAL VARIATION OF THE EGGS.

All of these as well as our previous experiments bring out the fact that the individual eggs vary a little in their reaction to the same solution. We are inclined to ascribe this result chiefly to a difference in the permeability of the individual eggs for bases, since it is not to be expected that the surface films of the individual eggs are exactly alike. Another source of variation seems to lie in the unequal distribution of the eggs in the solution, or at the bottom of the dish, whereby the chances for the equal diffusion of alkali or oxygen into the egg are diminished.

#### 10. APPLICATION OF THE METHOD TO THE EGGS OF OTHER FORMS.

The method of treating the unfertilized eggs with  $\text{NH}_4\text{OH}$  was also tried on the eggs of *Nereis* and of *Chaetopterus*. The eggs of the latter form suffer in the treatment but a small number were caused to segment. The eggs of *Nereis*, however, could be caused to segment and develop almost normally with this treatment. The method was not varied sufficiently to warrant us in giving details.

#### SUMMARY OF RESULTS.

1. The experiments show that the weak base  $\text{NH}_4\text{OH}$  is much more efficient for the causation of artificial parthenogenesis in *Arbacia* than the strong bases  $\text{KOH}$ ,  $\text{NaOH}$ , and tetraethylammoniumhydroxide. This fact corresponds with the observation made by the writer several years ago, that the weak acids (like the monobasic fatty acids or  $\text{CO}_2$ ) are much more efficient in the same process than the strong acids. The explanation given by him for the latter case seems to hold for the former, that only that part of the acid or base which is able to diffuse into the egg brings about artificial parthenogenesis.

2. The unfertilized eggs of *Arbacia* can be caused to develop into normal larvae by putting them into a mixture of 50 cc.  $m/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) + 0.3 cc.  $N/10$   $\text{NH}_4\text{OH}$  for twenty-five minutes, and afterwards into a neutral hypertonic solution, namely, 50 cc.  $m/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) + 8 cc.  $2\frac{1}{2}$   $m$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) for fifteen minutes (at a temperature of about  $22^\circ \text{C.}$ ) The eggs must be freed from sea water by repeated washing in a mixture of  $m/2$  ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) before they are put into the alkaline solutions. This method is almost as satisfactory as the butyric acid method.

3. The eggs treated for twenty-five minutes with  $\text{NH}_4\text{OH}$  form membranes in the hypertonic solution. They can also form membranes while in the  $\text{NH}_4\text{OH}$  solution, but in that case they must remain in the alkaline solution for a considerably longer time.

4. The effect of the  $\text{NH}_4\text{OH}$  can be inhibited or retarded by the addition of a few drops of  $\text{KCN}$  to the solution. Since our experiments indicate that only that part of the alkali can act which diffuses into the egg, and since it was shown formerly by Wasteneys and the writer that in spite of its low degree of dissociation  $\text{NH}_4\text{OH}$  is about half as effective for the increase in the rate of oxidations as  $\text{KOH}$ , the experiments suggest that in the egg of the sea urchin the oxidations are not confined to the external surface.

## DIE PHYSIKALISCHE NATUR BIOELEKTRISCHER POTENTIALDIFFERENZEN.\*

VON REINHARD BEUTNER<sup>1)</sup>.

*(Aus den Laboratorien des Rockefeller Institute for Medical Research,  
New York.)*

J. Loeb und der Verfasser haben vor kurzem in dieser Zeitschrift eine Abhandlung, betitelt: Über die Potentialdifferenzen an der unversehrten und verletzten Oberfläche tierischer und pflanzlicher Organe<sup>2)</sup>, veröffentlicht. In den nachstehenden Zeilen möchte ich eine physikalische Erklärung der in der genannten Arbeit gewonnenen experimentellen Ergebnisse zu geben versuchen. Meine Theorie muss sich dabei, soweit sie quantitativ ist, auf die Potentialdifferenzen an der Grenzfläche unversehrter pflanzlicher Organe und wässriger Lösungen beschränken; verletzte Flächen bieten deshalb komplizierte Verhältnisse, weil sie nicht homogen sind.

Der Sitz der bioelektrischen Potentialdifferenzen ist zweifellos an oder in den Membranen zu suchen, d. h. den nichtwässerigen Phasen des Gewebes, die die Zellen von angrenzenden wässrigen Flüssigkeiten trennen. Dies ist einerseits aus physikalischen Gründen von vornherein sehr wahrscheinlich und ergibt sich andererseits aus zahlreichen früheren Untersuchungen<sup>3)</sup>. Jede Schnittfläche durch lebendes Gewebe stellt gewissermassen ein Mosaik dar, indem teilweise die Membranen, teilweise die wässrigen Flüssigkeiten, die innerhalb und ausserhalb der Zellen sich befinden, zutage treten. Eine

\* Eingegangen am 26. August 1912.

<sup>1)</sup> Diese Abhandlung wurde dem 8. internationalen Kongress für angewandte Chemie (Washington und New York, September 1912) vorgelegt. Der Verfasser.

<sup>2)</sup> Diese Zeitschr. 41, 1, 1912.

<sup>3)</sup> Eine Literaturzusammenstellung gibt Cremer in Nagels Handbuch der Physiologie.

solche Schnittfläche bietet daher in Berührung mit wässerigen Lösungen komplizierte Phänomene; ebenfalls komplizierte Verhältnisse bietet die unverletzte tierische Haut, vielleicht auch zum Teil aus anderen Gründen. Die unverletzte Hülle pflanzlicher Organe dagegen kann als aus einer homogenen wasserunlöslichen Phase bestehend angesehen werden, die Potentialdifferenzen, die sich an derselben bei Berührung mit wässerigen Lösungen bilden, sind scharf definiert und momentan reversibel.

#### EXPERIMENTELLE TATSACHEN NACH DER FRÜHEREN ARBEIT.

Die experimentelle Untersuchung dieser Potentialdifferenzen, insbesondere ihre Abhängigkeit von der Zusammensetzung der wässerigen Lösung ist in der genannten Arbeit ausführlich beschrieben; sie verdient auch ein gewisses Interesse, denn diese Potentialdifferenzen sind wahrscheinlich gleicher oder ähnlicher Natur wie diejenigen an Membranen innerhalb des Gewebes überhaupt, die für elektrophysiologische Phänomene der verschiedensten Art verantwortlich, einer direkten physikalischen Untersuchung jedoch nicht zugänglich sind.

Ich möchte daher die untersuchten Potentialdifferenzen der Kürze halber als bioelektrische (abgekürzt b. P. D.) bezeichnen. Das für die folgende Untersuchung wichtigste Resultat der früheren Arbeit bezüglich derselben kann folgendermassen zusammengefasst werden:

Die b. P. D. (an der Grenze eines unverletzten Pflanzenteiles gegen die wässrige Lösung eines Alkali- oder Erdalkalisalzes) ist von der Konzentration der Lösung in der Weise abhängig, dass Verdünnung der Lösung dieselbe positiver macht. Alle Alkali- und Erdalkalisalze und auch alle anderen Elektrolyte zeigen ein solches Verhalten, soweit sie nicht stark giftig, d. h. chemisch verändernd auf die Oberfläche der Pflanze einwirken. Die Änderung der Potentialdifferenz ist in dem gleichen Sinne, diejenige an der Phasengrenze

Beliebiges Metall|Metallsalzlösung von wechselnder Konzentration.

Man kann also sagen, dass die b. P. D. für Kationen reversibel sind, und zwar in merkwürdigem Gegensatz zu

den bisher bekannten Potentialen für Kationen jeder Art. Die Grösse der Änderung ist in einem gewissen Konzentrationsbereich beinahe so gross wie nach der Nernstschen Formel für Potentialdifferenzen  $\left(E = \frac{RT}{nF} \ln C + \text{konst.}\right)$  zu erwarten ist.

Geht man von kleinen Konzentrationen der Salzlösung, etwa  $1/1000$  mol., aus und erhöht die Konzentration stufenweise in geometrischer Reihe, z. B. durch Verdoppelung, so findet man, dass die Veränderung der b. P. D. etwa  $0,058 \log 2$  ist, bis die Konzentration auf etwa  $1/50$  mol. gestiegen ist; über  $1/50$  mol. wird die Änderung erheblich kleiner, ist die Konzentration auf  $1/2$  bis  $1/1$  mol. gestiegen, so hat weitere Erhöhung keinen Einfluss. Mit steigender Konzentration strebt also die b. P. D. einem Grenzwert zu.

#### SCHEINBARE ABWEICHUNG VOM NERNSTSCHEN GESETZ BEI KLEINEN KONZENTRATIONEN.

Erniedrigt man die Konzentration unter  $1/1000$  mol., so findet man ebenfalls Abweichungen von der Nernstschen Formel. Diese Abweichungen sind indes nur scheinbar und dem Umstande zuzuschreiben, dass bei Konzentration von  $1/1000$  bis  $1/100\,000$  mol. die elektrolytischen Verunreinigungen des destillierten Wassers mit ins Spiel kommen. Das zu diesen Versuchen benutzte destillierte Wasser hatte schätzungsweise nach seiner Leitfähigkeit einen Ionen-Gehalt von  $1/10\,000$  bis  $1/100\,000$  mol. wechselnd. (Eine weitere sorgfältige Reinigung hatte keinen Zweck, da in Berührung mit der Blatt- oder Frucht-Oberfläche stets eine Erhöhung der Leitfähigkeit erfolgte.) Da bei den b. P. D. alle Elektrolyte in gleichem Sinne bei Konzentrationsänderung wirken, kann auch die Wirkung der verunreinigenden Ionen nicht vernachlässigt werden. Kontrolliert man durch Leitfähigkeitsbestimmungen die wahren Ionen-Konzentrationen, so findet man die Nernstsche Formel bis zu den höchsten Verdünnungen bestätigt, wie folgendes Beispiel zeigt.

Die Kette

Kalomel-Elektrode  $|1/10 \text{ n-KCl}| \text{Apfel} \underset{a}{|1/500 \text{ -KCl}|}$  Kalomel-Elektrode

zeigte die EMK  $0,064$  Volt ( $2'$  konstant). Wurde die  $1/500 \text{ -KCl}$ -

Lösung durch eine  $1/_{1000}$  n-KCl-Lösung ersetzt, so war die EMK, entsprechend der Änderung der b. P. D. bei  $a$  0,082 Volt, Differenz: 0,018 Volt, nach Nernstscher Formel berechnet:  $0,058 \log 2 = 0,0175$  Volt, also in vorzüglicher Übereinstimmung. Leitfähigkeitsbestimmungen ergaben, dass die Konzentration der Lösung durch Verunreinigungen nicht beeinflusst war. Wurde darauf die Konzentration der KCl-Lösung bei  $a$  durch 5fache Verdünnung auf  $1/_{5000}$  erniedrigt, so war die EMK: 0,110 Volt, Differenz gegen  $1/_{1000}$  n-KCl, 0,028 Volt, Nernsts Formel gibt  $0,058 \log 5 = 0,040$  Volt. Diese Abweichung wird verständlich durch Messung der Leitfähigkeit der (scheinbar)  $1/_{5000}$  n-KCl-Lösung.  $K_{21}$  gemessen 0,000 037 8, berechnet nach Kohlrausch 0,000 025 8. Nimmt man an, dass die verunreinigenden Ionen bezüglich Wanderungsgeschwindigkeit sich wie das KCl verhalten, so wäre die wahre Konzentration der  $1/_{5000}$  n-KCl-Lösung  $\frac{378}{258} \cdot \frac{1}{5000} = \frac{1}{2940}$ , Differenzen der EMKe bei  $1/_{1000}$  und  $1/_{2940}$ :

$0,058 \log 2,94 = 0,0272$  Volt. Gefunden: 0,028 Volt.

Diese scheinbare Abweichung hat hiernach mit besonderen Eigentümlichkeiten der b. P. D. nichts zu tun, ist vielmehr nur ein anderer Ausdruck der Tatsache, dass alle Kationen die b. P. D. im gleichen Sinne beeinflussen. Anders liegt es bei den Abweichungen von der Nernstschen Formel bei hohen Konzentrationen. Diese können nicht etwa durch Zurückgehen der elektrolytischen Dissoziation bei hohen Konzentrationen erklärt werden. Der Grenzwert der b. P. D. wird bei einer Konzentration von  $1/2$  mol. bis  $1/1$  mol. erreicht, auch bei weiterer Konzentrationssteigerung muss aber die absolute Ionenkonzentration offenbar trotz des Zurückgehens des Dissoziationsgrades steigen. Die Unveränderlichkeit der b. P. D. in diesem Gebiete zeigt, dass besondere Eigentümlichkeiten der Membran im Spiele sind.

#### ELEKTROMOTORISCHES VERHALTEN VON SYSTEMEN MIT ZWEI UNMISCHBAREN ELEKTROLYTISCHEN PHASEN.

Das Zustandekommen von Potentialdifferenzen, wie der b. P. D., kann physikalisch auf zwei verschiedenen Wegen erklärt werden. Auf jeden Fall ist es Voraussetzung, dass eine wasserunlösliche

Phase (in diesem Falle die Schale oder Membran des Pflanzenteiles) neben wässriger Lösung von Bedeutung ist. Es seien zunächst die allgemeinen physikalischen Tatsachen über das Zustandekommen von Potentialdifferenzen in Systemen mit zwei elektrolytischen Phasen besprochen.

Nernst hat zuerst das elektromotorische Verhalten von Systemen mit zwei unmischbaren Phasen experimentell und theoretisch untersucht. Gemeinsam mit Riesenfeld<sup>1)</sup> untersuchte er Anordnungen wie

Verdünnte wässrige Lösung eines beliebigen Salzes, z. B. $\text{AgNO}_3$	Phenol	Konzentrierte wässrige Lösung desselben Salzes ( $\text{AgNO}_3$ )
	1	2

Zwei Arten von Potentialdifferenzen sind in diesen Ketten zu unterscheiden.

1. „Diffusionspotentiale“ in Phenol. Diese kommen zustande, wenn die betreffenden Salze eine erhebliche Löslichkeit in Phenol besitzen, so dass die Stromleitung durch die Phenolschicht vermittels der gelösten Salze, resp. deren Ionen, vor sich geht. Unmittelbar an den Phasengrenzen 1 und 2 besteht nun Verteilungsgleichgewicht, das Phenol enthält bei 1 eine kleine, bei 2 eine grosse Konzentration an  $\text{AgNO}_3$ . In der Phenolschicht besteht also ein Konzentrationsabfall, die Änderung des Potentials längs desselben ist bekanntlich gegeben durch die Funktion:

$$\frac{u - v}{u + v} \frac{RT}{nF} \ln \frac{c_2}{c_1}$$

In wässrigen Lösungen sind  $u$  und  $v$  meist ungefähr gleich gross und deshalb die Diffusionspotentiale stets klein, in dem Phenol liegen ähnliche Verhältnisse vor. Nimmt man jedoch an, dass in manchen nicht wässrigen Phasen, wie z. B. den Membranen, erhebliche Unterschiede in der Wanderungsgeschwindigkeit der positiven und negativen Ionen bestehen, so könnte diejenige Art von Abhängigkeit der b. P. D. von Konzentrationen, wie sie hier beschrieben ist, auftreten. Ist z. B.  $v$  in der Membran gleich Null, so

<sup>1)</sup> Nernst und Riesenfeld, *Annal. d. Physik* 8, 600 und 609.

wäre das Diffusionspotential

$$= \frac{RT}{nF} \ln \frac{c_2}{c_1}$$

Ich möchte jedoch diese Deutung aus folgenden Gründen für nicht sehr wahrscheinlich halten.

Die Erklärung setzt voraus, dass die Stromleitung durch die zweite Phase (Membran) ausschliesslich durch die Salze, die aus der wässrigen Lösung in die Membran eintreten, vor sich geht. Nun sind, wie sich aus osmotischen Versuchen ergibt, die Membranen sehr schwer durchgängig für Salze. Nur äusserst geringe Salzspuren dringen also in die Membranen selbst unter den günstigsten Bedingungen, d. h. wenn die Salzlösung am konzentriertesten ist, ein. Die geringe Leitfähigkeit müsste durch diese Salzspuren bedingt sein und würde auf Null zurückgehen, wenn die Membran nur mit Wasser umspült ist. Dies ist im hohen Grade unwahrscheinlich, denn die Membran besitzt wahrscheinlich einen sehr geringen Gehalt an elektrolytischen Ionen, die unabhängig von den eingedrungenen ist, und da die Konzentration der letzteren sehr gering ist, nicht neben ihnen vernachlässigt werden kann.

Mit dieser Annahme ist es ferner kaum verständlich, weshalb die b. P. D. bei hoher Konzentration einem Maximum zustreben. Man sollte vielmehr das Umgekehrte erwarten, nämlich dass bei niedriger Konzentration ein Minimum besteht; die folgenden Überlegungen mögen dies näher erläutern.

Enthält die Membran keine anderen Elektrolyten als nur diejenigen, die durch Verteilungsgleichgewicht aus der wässrigen Lösung eingedrungen sind, so hätte die Kette

Konzentrierte wässrige Lösung Konz. $c_1$	Membran	Verdünnte wässrige Lösung Konz. $c_2$
---	---------	---

stets die EMK  $\frac{u-v}{u+v} \frac{RT}{nF} \ln \frac{c_1}{c_2}$ , welchen Wert  $c_1$  oder  $c_2$  auch annehmen; dies entspricht jedoch nicht den Tatsachen, da, wie gesagt, die EMK einem Maximum zustrebt.



Nimmt man nun an, dass die Membran eine geringe Menge eines in Wasser unlöslichen Elektrolyten enthält, so ist es offenbar von Bedeutung, ob dessen Konzentration neben der Konzentration der Elektrolyte, die auf beiden Seiten aus der wässrigen Lösung eindringen, vernachlässigt werden kann oder ob umgekehrt diese letztere Konzentration zu vernachlässigen ist. Bei einem bestimmt definierten Elektrolyt-Eigengehalt der Membran sei  $c_1$  und  $c_2$  relativ hoch; dann dringt auch aus den wässrigen Lösungen viel Elektrolyt in die Membran, und zwar so viel, nehmen wir an, dass der Elektrolyt-Eigengehalt daneben vernachlässigt werden kann; das Verhältnis der Elektrolyt-Konzentrationen in der linken und der rechten Grenzfläche der Membran (obige Kette) ist dann gleich,  $c_1 : c_2$  (nach dem Verteilungssatz, da in den Grenzflächen Gleichgewicht herrscht); die EMK obiger Kette also  $\frac{u - v RT}{u + v n F} \ln \frac{c_1}{c_2}$ . Sind dagegen  $c_1$  und  $c_2$  sehr klein gewählt, dringt also sehr wenig Elektrolyt aus der wässrigen Lösung in die Membran, so kann umgekehrt die eingedrungene Elektrolytmenge vernachlässigt werden, es befindet sich dann überhaupt kein elektromotorisch wirksames Konzentrationsgefälle in der Membran; die EMK der obigen Kette ist also Null. In einem Gebiete mittlerer Konzentration muss die EMK offenbar allmählich absinken; die Gesetzmässigkeiten, nach denen das geschieht, wären äusserst schwierig zu erforschen; diese Untersuchung ist aber auch unnötig, da, wie gesagt, die Tatsachen alle eben gemachten Folgerungen widerlegen: nicht bei grossen, sondern bei kleinen Werten von  $c_1$  resp.  $c_2$  ist die Änderung der EMK am grössten.

Diffusionspotentiale können die b. P. D. also wohl nur in zweiter Linie beeinflussen; eine ausschliessliche Erklärung erscheint auf dieser Basis unmöglich; es müssten weitere komplizierte Hilfsannahmen zu diesem Zweck gemacht werden, dabei hat die Annahme extrem differenzierter Ionen-Wanderungsgeschwindigkeiten schon an und für sich einen stark hypothetischen Charakter. Ich glaubte diese Erklärungsmöglichkeit hier nur deshalb diskutieren zu müssen, da physiologische Forscher den Verletzungsstrom auf diese Art zu erklären versucht haben. Auch diesen Theorien ist eine gewisse Unsicherheit kaum abzusprechen.

Die andere Art von Potentialdifferenzen, die in der Nernst-Riesenfeldschen Kette möglich ist, hat ihren Sitz an der Phasengrenze selbst.

Nernst<sup>1)</sup> selbst hat vor längerer Zeit gezeigt, dass Phasengrenzpotentiale zwischen elektrolytischen Leitern in derselben Weise wie Potentiale an Metall-Elektrolytgrenzen von der Ionen-Konzentration abhängig sind,  $= \frac{RT}{nF} \ln \frac{c_1}{c_2} + \text{konst.}$ , wo  $c_1$  und  $c_2$  Konzentrationen eines beliebigen beiden Phasen gemeinsamen Ion sind. Diese für die Elektrochemie fundamentale Tatsache beweist man am besten mit Hilfe einer fiktiven Kette wie

	Phase I	Phase II	
Metall	Salz des Metalles	Salz des Metalles	Metall
	enthaltend	enthaltend	
1	2	3	

Die Phase I und II sowie das Metall befinden sich im Gleichgewicht, und somit ist irgendeine chemische (oder physikalische) Reaktion in einer solchen Anordnung nicht möglich, auch nicht beim Stromfluss durch das System. Die Summe der 3 Potentialdifferenzen bei 1, 2 und 3 muss somit gleich Null sein.

$$\text{Da P. D. bei 1} = \frac{RT}{nF} \ln c_1 + \text{konst}_1$$

und P. D. bei 3  $= \frac{RT}{nF} \ln c_2 + \text{konst}_2$  in entgegengesetzter Richtung, so ergibt sich

$$\text{für P. D. bei 2} = \frac{RT}{nF} \ln \frac{c_1}{c_2} + \text{konst}_1 - \text{konst}_2.$$

In einer Kette wie der Nernst-Riesenfeldschen kommt diese Abhängigkeit des Phasengrenzpotentiales von der Konzentration nicht zum Vorschein, weil  $\text{AgNO}_3$  in Wasser und Phenol löslich ist und das Phenol keinen weiteren Elektrolyten enthält.

Für das Dissoziationsgleichgewicht im Wasser gilt:



Für das Dissoziationsgleichgewicht im Phenol gilt:



<sup>1)</sup> Nernst, Zeitschr. f. physikal. Chem. 9, 137, 1892.

Nach dem Verteilungssatz gilt, da an der Phasengrenze selbst stets Gleichgewicht herrschen muss,

$$\frac{c_{\text{AgNO}_3}}{C_{\text{AgNO}_3}} = K_3.$$

Division der beiden ersten Gleichungen gibt:

$$\frac{c_{\text{Ag}^+}}{C_{\text{Ag}^+}} \cdot \frac{c_{\text{NO}_3^-}}{C_{\text{NO}_3^-}} = \frac{K_1}{K_2} \cdot K_3 = K.$$

Da

$$c_{\text{Ag}^+} = c_{\text{NO}_3^-} \text{ und } C_{\text{Ag}^+} = C_{\text{NO}_3^-},$$

$$\frac{c_{\text{Ag}^+}^2}{C_{\text{Ag}^+}^2} = K, \quad \frac{c_{\text{Ag}^+}}{C_{\text{Ag}^+}} = \sqrt{K}.$$

Das Phasengrenzpotential ist also  $\frac{RT}{nF} \ln \sqrt{K} + \text{konst.}$ , d. h. von der  $\text{Ag}^+$ -Konzentration unabhängig. Eine Konzentrationserhöhung im Wasser bringt eben eine solche im Phenol mit sich, und das Verhältnis  $\frac{c_{\text{Ag}^+}}{C_{\text{Ag}^+}}$ , auf das es für die Potentialdifferenzen ankommt, bleibt unverändert. Dies ist auch der Fall, wenn der Elektrolyt in der einen Phase weitgehender als in der anderen dissoziiert ist, vorausgesetzt, dass das Verteilungsgesetz in der hier angewandten einfachen Form gilt. Anders verhält es sich, wenn die Ionenkonzentrationen der beiden aneinandergrenzenden Phasen sich nicht gegenseitig beeinflussen. Dies ist z. B. der Fall in der von Haber<sup>1)</sup> zuerst untersuchten Potentialdifferenz:



oder allgemein

Unlösliches Salz | Lösung eines Salzes mit einem gleichen Ion.

Die  $\text{Ag}^+$ -Konzentration in der Schicht des festen  $\text{AgCl}$  ist natürlich völlig unabhängig von der  $\text{Ag}^+$ -Konzentration der wässrigen Lösung. Nach der Nernstschen Formel ist die Potentialdifferenz gleich zu setzen:

$$\frac{RT}{F} \ln \frac{c_{\text{Ag}^+} \text{ im festen Ag}^+}{c_{\text{Ag}^+} \text{ in der wässrigen Lösung}} + \text{konst.}$$

<sup>1)</sup> Haber, Annal. d. Physik [4] 26, 947, 1908. An dieser Arbeit ist der Verf. dieser Arbeit ebenfalls beteiligt.

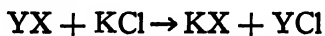
Da  $c_{Ag}$  im AgCl konstant bleibt, folgt somit, dass diese Potentialdifferenz sich wie eine solche von einer metallischen (Ag) Elektrode ändert, was sich experimentell bestätigen liess. Die Phasengrenzformel ist so einer direkten Prüfung zugänglich.

#### ANWENDUNG DER PHYSIKALISCHEN ERGEBNISSE AUF DIE B. P. D.

Die b. P. D. verhalten sich nun im Gebiete der kleinsten Konzentrationen  $\frac{n}{100}$  und weniger wie die Phasengrenzpotentiale vom Typus  $AgCl | Ag$ -haltige wässrige Lösung, im höchsten Konzentrationsgebiet (ca.  $\frac{n}{2}$  oder mehr) wie die Phasengrenzpotentiale der Nernst-Riesenfeldschen Kette. Das dazwischen liegende mittlere Konzentrationsgebiet verhält sich wie eine Übergangszone zwischen diesen beiden Extremen.

Eine befriedigende Erklärung dieses Verhaltens gelingt mit Hilfe der Annahme, dass die Membran einen Eigengehalt an Elektrolyten besitzt, d. h. wasserunlösliches Salz in homogener Mischung enthält. Ferner nehmen wir an, dass die Alkalisalze in der Membran eine kleine, aber endliche Löslichkeit besitzen.

Es sei das wasserunlösliche Salz in der Membran mit YX bezeichnet (Y' Anion, X' Kation), die wässrige Lösung enthalte KCl als Elektrolyt. Ferner müssen wir annehmen, dass YX mit dem in die Membran eingedrungenen KCl nach einer vollständig verlaufenen Gleichung reagiert, etwa so, dass elektrolytisch dissoziiertes KX und undissoziiertes YCl entsteht:

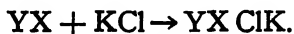


weitgehend  
dissoziiert

wenig  
dissoziiert

oder im Wasser leicht löslich, so dass  
es aus der Membran ausgelaugt wird.

Oder es könnte folgender Umsatz stattfinden:



Diese Additionsverbindung sei in die elektrolytischen Ionen  $Y X Cl'$  einerseits,  $K'$  andererseits dissoziiert (also in derselben Weise wie Platinchloriddoppelsalze). Beide Annahmen sind für unseren Zweck gleichwertig; sie sind geboten angesichts der Tatsache, dass die Reversibilität der b. P. D. gegenüber allen Kationen besteht.

Diese Annahme vom Umsatz gewisser Bestandteile des Gewebes

mit Salzen in wässriger Lösung ist übrigens durchaus nicht völlig neu, vielmehr in ähnlicher Weise auf Grund ganz anderer Beobachtungen vor langer Zeit schon von J. Loeb ausgesprochen worden. Loeb beobachtete, dass die Reizbarkeit sowie die rhythmische Bewegung von Muskeln in gemischten Salzlösungen eine reversible, von der Natur des Kations abhängige Änderung erfuhr; z. B. hebt Zusatz von sehr geringen Mengen von Ca zu Na-Lösungen die rhythmischen Bewegungen auf, ähnliche reversible Wirkungen lassen sich mit Anionen nicht beobachten. Loeb<sup>1)</sup> „stellte sich deshalb vor, dass die Metalle oder Metallverbindungen in den Geweben mit bestimmten organischen Stoffen, wie festen Säuren oder Eiweisskörpern, verbunden sind, und dass die Substitution eines Metalles für ein anderes die physikalischen Eigenschaften dieser Verbindungen ändert, z. B. die Oberflächenspannung, die Viscosität, das Wasserabsorptionsvermögen und den Aggregatzustand. Die Lebenserscheinungen und die Erscheinungen der Reizbarkeit hängen davon ab, dass gewisse Eiweissverbindungen oder Metallseifen in bestimmten Verhältnissen in den Geweben vorhanden sein müssen“. Diese Annahme wird durch die Resultate der vorliegenden Arbeit vollauf bestätigt, obgleich dieselben auf einem wesentlich anderen Gebiete liegen.

Ferner ist folgende für die Theorie vereinfachende Schlussfolgerung geboten. Bei genügend verdünnter Lösung ist, wie wir gesehen haben, die Nernstsche Formel für b. P. D. innerhalb eines weiten Konzentrationsgebietes in der gleichen Weise wie für Elektrodenpotentialdifferenzen gültig, nämlich

$$\text{b. P. D.} = \frac{RT}{nF} \ln c_{\text{wässr. Lösung}} + \text{konst.}$$

Dies ist mit der gemachten Annahme nur dann zu vereinigen, wenn der Umsatz nach irgendeinem der genannten Schema vollständig vor sich geht, das heisst von der Konzentration des Salzes in der wässrigen Lösung im wesentlichen unabhängig ist.

Nach dem Verteilungssatz ist andererseits die Konzentration des KCl in der Membran von der wässrigen KCl-Konzentration abhängig, und wir nehmen an, dass das Verteilungsgesetz in der ein-

<sup>1)</sup> Loeb, *Dynamik der Lebenserscheinungen*. S. 120.

fachsten Form gilt, dass also Proportionalität der wässerigen und der Membrankonzentration besteht. Bei kleinen wässerigen Konzentrationen ist die KCl-Konzentration in der Membran so klein, dass sie neben der  $K^+$ -Konzentration, die durch den Elektrolyteigengehalt der Membran bedingt ist, vernachlässigt werden kann. Offenbar ist hiermit die Bedingung einer Veränderlichkeit der b. P. D. nach der Art der Potentialdifferenz  $AgCl | Ag$ -Lösung gegeben. Bei den höchsten wässerigen Konzentrationen dagegen steigt die KCl-Konzentration der Membran so weit, dass nunmehr umgekehrt der  $K^+$ -Eigengehalt der Membran vernachlässigt werden kann; das würde offenbar also eine Veränderlichkeit der b. P. D. nach Art der Potentialdifferenz  $AgNO_3$  in Phenol  $| AgNO_3$  in Wasser zur Folge haben, wie es auch tatsächlich beobachtet wird.

#### ANALYTISCHE FORMULIERUNG DER BESCHRIEBENEN HYPOTHESE.

Zur Darstellung der gegenseitigen Abhängigkeit der Ionenkonzentrationen in Membran und wässriger Lösung benutzen wir die üblichen Vorstellungen der elektrolytischen Dissoziation und des beweglichen Gleichgewichtes.

Membran	Wasser	c bezeichne Konzentrationen in wässriger Lösung
$KX = K^+ + X^-$	$KCl = K^+ + Cl^-$	C Konzentrationen in der Membran.
$KCl = K^+ + Cl^-$		

Wir nehmen an, dass für die elektrolytische Dissoziation des KCl in Wasser und Membran das Massenwirkungsgesetz in der gleichen Weise angesetzt werden kann, nämlich:

$$c_K \cdot c_{Cl'} = K_1 c_{KCl}^n$$

in der wässerigen Lösung, also entsprechend

$$C_K \cdot C_{Cl'} = K_2 C_{KCl}^n$$

in der Membran.

(Die Konstanten  $K_1$  und  $K_2$  sollen nicht gleich sein wohl aber die Exponenten von  $c_{KCl}$  und  $C_{KCl}$ ).

Das Verteilungsgesetz setzen wir an in der einfachen Form:

$$\frac{C_{KCl}}{c_{KCl}} = K_3.$$

Division der beiden ersten Gleichungen und Substitution mittels der dritten ergibt:

$$\frac{c_K}{C_K} = \frac{K_1}{K_2} \cdot \frac{c_{Cl'}}{C_{Cl'}} = \frac{1}{K} \frac{c_{Cl'}}{C_{Cl'}} \quad \dots \quad 1)$$

1) Form des Massenwirkungsgesetzes für starke Elektrolyte nach van't Hoff und nach Rudolphi.



5 nimmt dann die Form an:

$$b. P. D. = \frac{RT}{nF} \ln \frac{C_X}{c} + \text{konst.}$$

oder

$$= \frac{RT}{nF} \ln \frac{1}{c} + \text{konst.}',$$

d. h. die b. P. D. ändert sich mit  $c$  wie eine Elektrodenpotentialdifferenz.

2. Sehr grosse wässrige Konzentrationen. In diesem Falle kann umgekehrt  $\left(\frac{C_X}{2c}\right)^2$  neben  $K$  vernachlässigt werden. Die Formel 5 nimmt die einfache Gestalt an:

$$b. P. D. = \frac{RT}{nF} \ln \sqrt{K} + \text{konst.},$$

d. h. bei hohen Konzentrationen ist die b. P. D. von  $c$  unabhängig.

Das Experimentum crucis für die Brauchbarkeit unserer Theorie bieten jedoch die mittleren Konzentrationen, denn offenbar können die Grenzbedingungen auch durch eine einfachere empirische Gleichung zum Ausdruck gebracht werden. Die Art und Weise, mit der die b. P. D. vom Grenzwert bei hohen Konzentrationen absinkt, wenn die Lösung stufenweise verdünnt wird ist experimentell mit den Forderungen der Theorie zu vergleichen. Zwecks empirischer Prüfung der Gleichung 5 müssen die Konstanten  $C_X$  und  $K$  zunächst empirisch bestimmt werden. Eine einzige Bestimmung ist, wie die folgenden Ableitungen zeigen, hierfür genügend. Mathematisch am einfachsten ist es, als Differenz diejenige bei  $C = 1/500$  und der Grenz-EMK bei sehr grossem  $c$  zu wählen. Es gilt dann bei Zimmertemperatur:

$$b. P. D. \text{ bei } c=1/500 = 0,058 \log (250 C_X + \sqrt{250^2 C_X^2 + K}) + \text{konst.}$$

(in Volt)

$$b. P. D. \text{ limit} = 0,058 \log \sqrt{K} + \text{konst.}$$

$$\text{Differenz: } b. P. D. \text{ bei } c=1/500 - b. P. D. \text{ limit} = 0,058 \log \frac{250 C_X + \sqrt{250^2 C_X^2 + K}}{\sqrt{K}}.$$

Diese Differenz setzen wir gleich  $0,058 \log 1/m$ , wo  $m$  also eine experimentell zu bestimmende Grösse ist.

Es folgt

$$250 C_X + \sqrt{250^2 C_X^2 + K} = \frac{1}{m} \sqrt{K}.$$

Auflösung dieser Gleichung nach  $\sqrt{K}$  ergibt:

$$\sqrt{250^2 C_X^2 + K} = \frac{1}{m} \sqrt{K} - 250 C_X$$

quadriert:

$$250^2 C_X^2 + K = \frac{1}{m^2} K - 500 \frac{1}{m} \sqrt{K} C_X + 250^2 C_X^2,$$

mit  $\sqrt{K}$  dividiert:



$$\begin{aligned}\sqrt{K} &= \frac{1}{m^2} \sqrt{K} - 500 \frac{1}{m} C_X \\ - \sqrt{K} + \frac{1}{m^2} \sqrt{K} &= 500 \frac{1}{m} C_X \\ \sqrt{K} &= \frac{500 C_X}{m \left( \frac{1}{m^2} - 1 \right)}.\end{aligned}$$

Berücksichtigt man, dass  $\log 1/m$  gewöhnlich etwa gleich 2 ist,  $\frac{1}{m^2}$  also ca. 10000, so kann statt  $\frac{1}{m^2} - 1$  geschrieben werden  $\frac{1}{m^2}$ . Für  $\sqrt{K}$  ergibt sich dann näherungsweise:

$$\sqrt{K} = 500m C_X.$$

Dieser Wert in Gl. 5 substituiert gibt für

$$\text{b. P. D.} = \frac{RT}{nF} \ln \left( \frac{C_X}{2c} + \sqrt{\frac{C_X^2}{4c^2} + 500^2 m^2 C_X^2} \right) + \text{konst.}$$

Die Differenz zweier beliebigen EMKe, die sich nur durch die Konzentrationen  $c_1$  resp.  $c_2$  an der Grenze der Membran unterscheiden, ist also

$$E_{c_1} - E_{c_2} = 0,058 \log \frac{\frac{C_X}{2c_1} + \sqrt{\frac{C_X^2}{4c_1^2} + 500^2 m^2 C_X^2}}{\frac{C_X}{2c_2} + \sqrt{\frac{C_X^2}{4c_2^2} + 500^2 m^2 C_X^2}}.$$

Wie man sieht, hebt sich  $C_X$  aus dem Quotient hinter dem  $\log$  heraus; wird der Quotient ausserdem mit 2 erweitert, so ergibt sich

$$\begin{aligned}E_{c_1} - E_{c_2} &= 0,058 \log \frac{\frac{1}{c_1} + \sqrt{\frac{1}{c_1^2} + 1000^2 m^2}}{\frac{1}{c_2} + \sqrt{\frac{1}{c_2^2} + 1000^2 m^2}} \\ &= 0,058 \log \frac{\frac{1}{c_1} \cdot (1 + \sqrt{1 + 10^6 m^2 c_1^2})}{\frac{1}{c_2} \cdot (1 + \sqrt{1 + 10^6 m^2 c_2^2})} \\ E_{c_1} - E_{c_2} &= 0,058 \log \frac{c_2}{c_1} - 0,058 \log \frac{1 + \sqrt{1 + 10^6 m^2 c_2^2}}{1 + \sqrt{1 + 10^6 m^2 c_1^2}}\end{aligned}$$

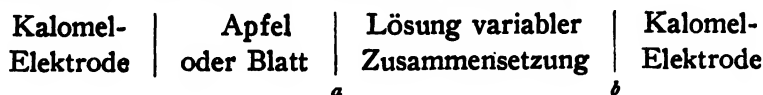
$m$  war oben definiert durch die Beziehung

$$\log \frac{1}{m} = \frac{E_c - 1/500 - E_{\text{limit}}}{0,058}.$$

#### EXPERIMENTELLE PRÜFUNG DER THEORIE.

Diese Formel gilt, wenn KCl als Elektrolyt zugegen ist oder auch bei irgend einem anderen binären einwertigen Elektrolyten. Für

zweiwertige Elektrolyte wie  $\text{CaCl}_2$  lässt die Theorie eine kompliziertere Beziehung voraussehen. Die experimentelle Prüfung geschieht durch Messung der EMK von Ketten wie



In dieser Kette ist ausser der b. P. D. bei  $a$  auch das Diffusionspotential bei  $b$  von der Konzentration der variablen Lösung abhängig. Ist KCl als Elektrolyt in der variablen Lösung sowie in der Kalomel-Elektrode (konstant  $1/10n$ ), so kann das Diffusionspotential bei  $b$  als unveränderlich angesehen werden, und es sind deshalb solche Versuche zur Prüfung der Theorie besonders geeignet.

1. Versuchreihe beim Apfel. (KCl als Elektrolyte.)

$$\text{Gemessen bei } c = \frac{1}{500} n\text{-KCl:} \quad 0,0874 \text{ Volt}$$

$$\text{" " } c = \frac{1}{n} \text{ oder } \frac{1}{2} n\text{-KCl:} \quad -0,0220 \text{ "}$$

Dies kann als dem Grenzwert der b. P. D. entsprechend angesehen werden.

$$\text{Es ist somit } \log \frac{1}{m} = \frac{0,1904}{0,058} = 1,8862,$$

$$\log m^2 = -3,7724$$

$$10^8 m^2 = 169$$

$$E_{c_2} - E_{c_1} = 58 \log \frac{c_2}{c_1} - 58 \log \frac{1 + \sqrt{1 + 169 c_2^2}}{1 + \sqrt{1 + 169 c_1^2}}.$$

(in Millivolt)

Konzentrations- Intervall	Berechnet (Millivolt)	Beobachtet bei		Mittel (Millivolt)
		zunehmender	abnehmender	
Konzentration				
1/1 bis 1/2	1,3	—	1,0	1,0
1/2 " 1/4	3,8	6,7	3,0	4,9
1/4 " 1/8	7,0	9,6	6,0	7,8
1/8 " 1/16	11,4	8,9	11,3	10,1
1/10 " 1/20	12,8	13,2	12,8	13,0
1/20 " 1/40	15,8	15,6	12,8	14,4
1/40 " 1/80	17,0	16,0	14,7	15,3
1/2 " 1/10	13,5	19,0	21,0	20,0
1/4 " 1/20	22,5	15,5	26,5	21,0
1/8 " 1/40	31,2	32,0	30,0	31,0
1/16 " 1/80	36,7	38,6	35,0	36,8

Wie man sieht, sind bei Änderung der Konzentration in entgegengesetzter Richtung die Änderungen der EMK nicht völlig gleich, besonders bei hohen Konzentrationen besteht diese Unsicherheit. Angesichts dieser mangelhaften Reversibilität kann die Übereinstimmung mit der Theorie als befriedigend angesehen werden, die Abweichungen der beobachteten Mittelwerte von den berechneten sind nur in einem Falle grösser als 2 Millivolt (Intervall  $1/2$  bis  $1/10$ ), in den übrigen 10 Fällen kleiner. Eine grössere Genauigkeit als 2 Millivolt kann bei solchen Messungen nicht erzielt werden. (Die Messung geschah n. b. mit einem empfindlichen Spiegelelektrometer.)

Eine weitere Versuchsreihe wurde an einem anderen Apfel mit KCl ausgeführt, wobei die Konzentrationsintervalle andere waren.

$$\text{Gemessen bei } c = \frac{1}{500} \text{ KCl: } 0,0750 \text{ Volt;}$$

$$\text{„ „ } c = \frac{1}{1} \text{ KCl: } -0,0294 \text{ „}$$

$$\text{Es ist somit } \log \frac{1}{m} = \frac{0,1046}{0,058} = 1,8034,$$

$$10^0 m^2 = 247,28.$$

Konzentrations- Intervall	Berechnet in Millivolt	Beobachtet in Millivolt
$1/500$ n bis $1/250$ n	17,5	17,0
$1/250$ n „ $1/125$ n	17,0	15,2
$1/125$ n „ $1/25$ n	38,5	36,3
$1/50$ n „ $1/25$ n	16,0	16,0
$1/25$ n „ $1/5$ n	23,5	27,1
$1/5$ n „ $1/1$ n	6,5	8,0
$1/1$ n „ $2 \ 1/2$ n	1,0	1,0 (in entgegengesetzter Richtung)

Auch bei diesen Versuchen ist die Übereinstimmung befriedigend.

Messungen der gleichen Art wie die eben beschriebenen mit dem Blatt einer Gummipflanze (*Ficus elastica*) sind in der genannten früheren Arbeit gegeben. Ich möchte auch für diese Messungen die hier abgeleitete Formel prüfen.

Konzentrations- Intervall	Beobachtet			Mittel	Be- rechnet
	I	II	III		
1/1250 bis 1/250	31	33	35	33	40,0
1/250 " 1/50	30	32	31	31	36,5
1/50 " 1/10	23	24	21	23	18,5

Zur Ausführung der Berechnung war die Kenntnis des Grenzwertes der Kraft, welcher bei noch höherer Konzentration erreicht wird, erforderlich. Er wurde in besonderen Versuchen gefunden:

Konzentrations-Intervall:

$1/_{10}n$  bis  $1/_{2}n$

5 Millivolt

Die Berechnung ergab:

4,5 Millivolt

$1/_{2}n$  "  $5/_{2}n$

2 "

1 "

Was die Berechnung für diese Beispiele betrifft, so wurde hier so verfahren, dass die Grösse  $m$  definiert wurde durch die Beziehung

$$\log \frac{1}{m} = \frac{E_{c=1/250} - E_{\text{limit}}}{0,058}.$$

Entsprechend lautet die Hauptformel:

$$E_{c_2} - E_{c_1} = 58 \log \frac{c_2}{c_1} - 58 \log \frac{1 + \sqrt{1 + 500^2 m^2 c_2^2}}{1 + \sqrt{1 + 500^2 m^2 c_1^2}}.$$

(in Millivolt)

In derselben Weise sind auch Versuche mit NaCl-Lösungen verschiedener Konzentration aus der früheren Arbeit durchgerechnet worden; hierbei ist jedoch zu beachten, dass in der entsprechenden Kette

Kalomel- Elektrode	Blatt des Gummi- baumes	NaCl-Lösung wechsell- der Konzentration	Kalomel- Elektrode
	a		b+
			→

auch das Diffusionspotential bei  $b$  sich mit der Konzentration der variablen Lösung ändert. (Die Kalomel-Elektroden waren bei diesen Versuchen mit  $1/_{10}$  n-NaCl-Lösung gefüllt.) Nach einer bekannten Formel kann für Verfünffachung der Konzentration die

Änderung des Diffusionspotentials gleich  $\frac{u-v}{u+v} \cdot 0,058 \log 5$  gesetzt werden, diese Grösse kann für NaCl-Lösungen nicht so wie bei KCl vernachlässigt werden, da  $u$  und  $v$  nicht völlig gleich sind; es ergibt sich vielmehr hierfür der Wert 0,008 Volt; die Änderung ist

in dem Sinne, dass die variable Lösung mit steigender Verdünnung negativer gegen die Kalomel-Elektrode wird (da das negative Ion Cl eine grössere Wanderungsgeschwindigkeit als das positive Na hat). Somit ist die Änderung in dem gleichen Sinne wie die Gesamtwirkung der Konzentrationsänderung: bei Verdünnung wird die rechte Seite der obigen Kette positiver, und es ist von der gemessenen Gesamtdifferenz bei Verfünfachung der Konzentration 0,008 Volt abzuziehen, um die Differenz der b. P. D. zu erhalten.

Zur Berechnung möchte ich die zweite NaCl-Versuchsreihe, die in der genannten Arbeit mitgeteilt ist, wählen, es war gefunden:

Konzentrationsintervall	Differenz gefunden	Nach Abzug des Differenzpotentials	Berechnet
1/1250 bis 1/250	38	30	37.5
1/250 " 1/50	36	28	37.0
1/50 " 1/10	24	18	14.5

In einer anderen (nicht mitgeteilten) Versuchsreihe, für die das Konzentrationsintervall  $1/50$  bis  $1/10$  dieselbe Differenz der Kraft gab wie oben, ergab sich für

Konzentrationsintervall	Differenz gefunden	Nach Abzug des Differenzpotentials	Berechnet
1/10 bis 1/2	13	5	3.5
1/2 " 5/2	11	3	1.0

Für die Berechnung wurde in diesem Falle gesetzt

$$\log \frac{1}{m} = \frac{0,052}{0,058} = 0,8966, \text{ also } 500^2 m^2 = 4025$$

$$E_{c_2} - E_{c_1} = 58 \log \frac{c_2}{c_1} - 58 \log \frac{1 + \sqrt{1 + 4025 c_2^2}}{1 + \sqrt{1 + 4025 c_1^2}}.$$

Die Übereinstimmung mit den theoretischen Werten ist bei diesen letzten Versuchen weniger gut als bei den ersten. Ich glaube das dadurch erklären zu können, dass diese letzten Versuchsreihen nicht an ein und demselben Objekt hintereinander durchgeführt wurden, somit von Unsicherheiten durch Verschiedenheit des biologischen Materiales weniger frei sind. Die NaCl-Versuche sind ausserdem mit den Unsicherheiten behaftet, die die Berechnung von Diffusionspotentialen mit sich bringt.

Zur richtigen Würdigung der vorliegenden Ergebnisse sei nochmals auf die Einzelannahmen hingewiesen, die zur Ableitung der Formeln nötig waren. Diese waren:

1. Gültigkeit des Verteilungssatzes (für Membran: Lösung) in der einfachsten Form.

2. Ähnlichkeit der elektrolytischen Dissoziation in Membran und wässriger Lösung.

Ohne diese Annahmen steigen die mathematischen Schwierigkeiten alsbald zum fast Unüberwindbaren, und es scheint uns dann eben unmöglich, überhaupt zu quantitativen Beziehungen zu gelangen. Man wird zugeben, dass diese Hilfsannahmen ihrer Natur nach plausibel und in befriedigender Annäherung durch das Experiment bestätigt sind.

#### BEZIEHUNGEN ZU NERNSTS THEORIE DER REIZUNG.

In der früheren mehrfach erwähnten Arbeit, die der Verfasser mit Jacques Loeb ausgeführt hat, wurde bereits gezeigt, dass die Potentialdifferenzen an der Schnittfläche pflanzlicher Gewebe qualitativ, aber nicht quantitativ sich ebenso verhalten wie solche an unverletzten Oberflächen; die Änderung der Potentialdifferenz mit der Konzentration der ableitenden Lösung erfolgt in demselben Sinne, jedoch in erheblich kleineren Beträgen. An der Hand der im Eingang dieser Arbeit gemachten Bemerkung, dass die Schnittfläche sich elektrisch wie ein Mosaik verhält, erscheint diese Beobachtung erklärlich. An der Grenze der durchschnittenen Membranteile wirkt die Konzentrationsänderung wie an der unverletzten Rinde, an denjenigen Stellen jedoch, wo ableitende Lösung und Saft zusammenkommen, ist kein Einfluss der Konzentration auf die Potentialdifferenz vorhanden. Es sind also in diesen Versuchen gewissermassen zwei Ketten parallel geschaltet, solche mit wahren b. P. D. und solche mit Diffusionspotentialen; der Gesamteffekt muss also offenbar eine kleinere Konzentrationsveränderlichkeit als bei den b. P. D. sein, wie es auch tatsächlich beobachtet wird.

Möglich wäre es auch, die Erscheinungen an der Schnittfläche mit der Annahme zu deuten, dass die elektromotorisch wirksame Membran mit einer fest anhaftenden wässrigen Phase bedeckt ist,

die Konzentration der ableitenden Flüssigkeit kann sich dann nur durch Diffusion durch diese Schicht hindurch bemerkbar machen.

Tierische Gewebe der verschiedensten Art verhalten sich ähnlich den verletzten Pflanzenteilen, wie auch schon in der früheren Arbeit gezeigt wurde. Ich möchte an dieser Stelle noch einmal auf die interessanten Versuche von McDonald<sup>1)</sup> und einen sich daraus ergebenden Zusammenhang mit der Nernstschen Theorie der Reizung hinweisen.

McDonald hat analoge Versuche mit Warmblüternerven ausgeführt, wie sie von Herrn Dr. Loeb und mir mit Pflanzenteilen beschrieben wurden. Freilich ist hiermit weder die von uns erreichte Präzision zu konstatieren, und es konnte deshalb auch nicht die hier gegebene physikalische Erklärung gewonnen werden. Die wässrige Lösung, die an der äusseren Oberfläche der Nerven haftet, wird durch Einlegen in Lösungen verschiedener Konzentration in ihrer Konzentration geändert, und damit die EMK, die man bei Ableiten von Oberfläche und Querschnitt misst (gewöhnlich als Verletzungsstrom bezeichnet). Bekanntlich ist die Oberfläche positiv gegen den Querschnitt und sie wird um so positiver, je verdünnter die Spüllösung ist, in die der Nerv gelegt war und welche also an der Oberfläche haftet. Also selbst bei diesen Objekten wirkt die Konzentration in dem gleichen Sinne auf die b. P. D. Ist es hiernach nicht berechtigt, die Beziehung zwischen b. P. D., wie sie oben entwickelt wurde, als charakteristisch für Membranen verschiedenster Art anzusehen, wenigstens in gewisser Annäherung?

Mit zahlreichen elektro-physiologischen Erscheinungen müssen die b. P. D. offenbar im Zusammenhang stehen. Besonders interessant scheinen mir die Beziehungen zu der Nernstschen Theorie der Reizung. Die unter dieser Bezeichnung gewöhnlich zusammengefassten Gesetzmässigkeiten sind durch ein besonders solides Fundament von Tatsachen begründet. Nernst<sup>2)</sup> hat gezeigt, dass die Erregung eines und desselben Muskels unter dem Einfluss von oszillatorischem oder alternierendem Stromfluss bei wechselnden elektrischen Bedingungen immer dann eintritt, wenn der Stromfluss

<sup>1)</sup> Proc. Roy. Soc. 67, 310, 1900.

<sup>2)</sup> Sitzungsber. d. preuss. Akad. 1, 1, 1908. Siehe auch Arch. f. d. ges. Physiol. 122, 307, 1908.

(oder die Entladung) eine gewisse maximale Konzentrationsveränderung an Phasengrenzen zustande bringen kann. Zur Berechnung dieser Konzentrationsänderung (an der Phasengrenze zweier Elektrolyte) können dieselben Gesetzmässigkeiten wie an einer Metallelektrode angenommen werden, z. B. als Funktion der Stromstärke und Wechselzahl eines Wechselstromes ist die Konzentrationsänderung gegeben als konst.  $\frac{i}{\sqrt{m}}$  wo  $i$  die Stromstärke,  $m$  die Perio-

denzahl des Wechselstromes bedeutet. In der Tat zeigt sich, dass für alle Wechselströme, die gerade die Reizung eines und desselben Nervmuskelpreparates hervorrufen können, dieser Quotient konstant ist. — Wie wir gesehen haben, bedingen Konzentrationsänderungen Änderungen der Potentialdifferenz. Hiernach scheint es bei der Reizung auf eine gewisse maximale Polarisation an der Grenzfläche Membran | Lösung anzukommen. Dass diese Polarisation durch Vermittlung einer damit verknüpften Änderung der Oberflächenspannung in die Kette der Vorgänge eingreifen kann, soll hier nur angedeutet werden.

Bemerkenswert ist, dass der Elektrolytgehalt der meisten Körperflüssigkeiten konstant  $\frac{1}{8}$  molekular ist. Ein Blick auf die Tabelle auf S. 401 zeigt, dass diese Konzentration für die b. P. D. insofern eine besondere Bedeutung hat, als Konzentrationssteigerung nur mehr eine sehr kleine Änderung hervorbringen kann, Konzentrationserniedrigung dagegen eine sehr viel grössere. Dies scheint mir nach dem oben erklärten Zusammenhang zwischen Polarisation und Reizung mit der Tatsache verknüpft zu sein, dass gleiche Stromstösse in verschiedener Richtung sehr verschiedene Reizeffekte haben. Bekanntlich ist die Reizung bei Schliessung eines Stromes sehr viel stärker an der Kathode als an der Anode, bei Öffnung umgekehrt. Die Konzentrationsverschiebungen in entgegengesetzten Richtungen bringen eben sehr verschiedene Änderungen der b. P. D. hervor. Ubrigens würde dies auch der Fall sein, wenn für die b. P. D. eine einfache logarithmische Konzentrationsunabhängigkeit bestände, da der Stromstoss die Absolutwerte der Konzentration nicht ihr Verhältnis ändert. Bei Gültigkeit meiner Formel ist die einseitige Wirkung der Konzen-



trationsänderung jedoch noch besonders ausgeprägt und am meisten für die Salzkonzentrationen, die in den Körpersäften vorkommen.

Eine andere Erscheinung, die vielleicht in demselben Zusammenhange eine Erklärung ist, ist die folgende. Overton<sup>1)</sup> erwähnt, dass die Reizbarkeit von Muskeln in hyperisotonischen Lösungen ( $> 1/8$  mol.) schneller verloren geht als in hypoisotonischen ( $< 1/8$  mol.), obgleich in beiden Fällen schädigende Einflüsse sich bemerkbar machen. Die Tabelle auf S. 401 zeigt, dass bei Konzentrationen, die wesentlich höher als  $1/8$  mol. sind, die b. P. D. sich nahezu in dem konzentrationsunabhängigen Gebiete befindet, es ist keine Polarisation möglich und damit der Erregungsvorgang gehemmt. Es soll indes nicht geleugnet werden, dass eine Konzentrationsänderung in diesen Fällen auch durch osmotische oder chemische Vorgänge Veränderungen im Gewebe hervorrufen kann, die ihrerseits die Reizbarkeit beeinflussen.

Hiermit scheinen mir zum ersten Male gewisse Anhaltspunkte gewonnen zu sein für das Verständnis der Tatsache, dass die Elektrolytkonzentration der Körpersäfte bei höheren Organismen konstant etwa  $1/8$  g mol. sein muss.

#### ZUSAMMENFASSUNG.

Potentialdifferenzen an der Berührungsfläche:

Pflanzenteil | wässrige Lösung eines beliebigen Elektrolyten ändern sich, wie Herr Dr. Loeb und der Verfasser gezeigt haben, reversibel mit der Konzentration der wässrigen Lösung in dem Sinne, dass steigende Verdünnung die Lösung positiver macht.

1. Zur Darstellung der Veränderlichkeit dieser Potentialdifferenz mit der Konzentration wird die Beziehung abgeleitet:

$$\text{Pot.-Diff. 1} - \text{Pot.-Diff. 2} = 58 \log \frac{c_1}{c_2} - 58 \log \frac{1 + \sqrt{1 + 10^6 m^2 c_1^2}}{1 + \sqrt{1 + 10^6 m^2 c_2^2}}$$

Millivolt

wo m durch die Beziehung gegeben ist:

$$\log \frac{1}{m} = \frac{\text{Grenzwert d. Pot.-Diff.} - \text{Pot.-Diff. für } c = 1/500}{58}$$

<sup>1)</sup> Arch. f. d. ges. Physiol. 42, 346.

Der Ableitung dieser Formel liegt die Vorstellung zugrunde, dass ein vollständiger Umsatz zwischen einem Bestandteile der Membran und dem Elektrolyten der Lösung stattfindet, wobei ein wasserunlösliches Salz gebildet wird, das in der Membran in homogener Mischung enthalten ist (Eigengehalt der Membran). Ausserdem sei der wässrige Elektrolyt infolge minimaler Löslichkeit als solcher in der Membran enthalten; für das Verhältnis der Konzentrationen Elektrolyt in Membran: Elektrolyt in Wasser wird das Verteilungsgesetz in der einfachsten Form angesetzt.

2. Die Formel lässt sich experimentell durch Messungen am Apfel gut bestätigen (siehe Tabellen S. 401 u. ff.). (Bei hohen Konzentrationen erreicht die Potentialdifferenz einen Grenzwert, bei kleinen Konzentrationen folgt sie einer einfachen logarithmischen Gesetzmässigkeit; das Verhalten bei mittleren Konzentrationen kommt durch die Formel gut zum Ausdruck.)

3. Im Zusammenhang mit Nernsts Theorie der Reizung erklärt dieses Verhalten von bioelektrischen Potentialdifferenzen einige electrophysiologische Beobachtungen, wenn man annimmt, dass die mit dem elektrolytischen Konzentrationsänderungen verknüpften Änderungen der Potentialdifferenz für die Erregung massgebend sind. Die Beobachtungen sind: a) verschiedener Reizwert entgegengesetzt gerichteter, gleich starker Stromstösse, b) erregungshemmende Wirkung konzentrierter Salzlösungen. Bei dieser Erklärung ist noch die Annahme gemacht, dass die innerhalb der Gewebe befindlichen Membranen sich ähnlich den hier untersuchten verhalten; diese Annahme lässt sich durch gewisse Beobachtungen begründen.

## UNTERSUCHUNGEN ÜBER PERMEABILITÄT UND ANTAGONISTISCHE ELEKTROLYTWERKUNG NACH EINER NEUEN METHODE.\*

VON JACQUES LOEB.

(Aus den Laboratorien des Rockefeller Institute for Medical Research,  
New York.)

### I.

Vor 12 Jahren führte ich den Begriff der physiologisch äquilibrierten Salzlösung ein<sup>1)</sup>. Als Beispiele derartiger Lösungen dürfen das Seewasser oder eine Mischung von  $\text{NaCl} + \text{KCl} + \text{CaCl}_2$  in dem Verhältnis dienen, in dem diese Salze im Seewasser enthalten sind. Die Notwendigkeit der Einführung dieses Begriffes gründete sich auf die Tatsache, dass Lösungen von einzelnen Salzen, z. B.  $\text{NaCl}$ , in der Konzentration, in der dieses Salz im Seewasser enthalten ist, für die meisten Seetiere giftig sind, während der Zusatz von kleinen Mengen gewisser anderer Salze, besonders der Salze mit zweiwertigem Kation, diese Giftigkeit verringert<sup>2)</sup>. Physiologisch äquilibrierte Salzlösungen sind nun solche, in denen die giftigen Wirkungen, die jedes Salz hätte, wenn es allein in Lösung wäre, sich gegenseitig aufheben; in denen m. a. W. der gegenseitige Antagonismus der einzelnen Bestandteile möglichst vollständig ist. Die Frage entstand, was der Mechanismus dieser antagonistischen Salzwirkung ist.

Vor 7 Jahren stellte ich die Hypothese auf, dass das Wesen der antagonistischen Salzwirkungen darauf beruht, dass die Lösung eines einzelnen Salzes, wenn seine Konzentration in der Lösung hoch genug ist, die Durchlässigkeit der Zellmembranen (oder der

\* Eingegangen am 24. Oktober 1912.

<sup>1)</sup> Amer. Journ. of Physiol. 3, 434, 1900. Abgedruckt in „Untersuchungen über künstliche Parthogenese“, S. 99. Leipzig 1906.

<sup>2)</sup> Amer. Journ. of Physiol. 3, 327, 1900; Arch. f. d. ges. Physiol. 88, 68, 1901; Amer. Journ. of Physiol. 6, 411, 1902.

äusseren Membran von Eiern) erhöht und dass infolgedessen das Salz in die Zellen eindringt und die dort stattfindenden chemischen Reaktionen oder die physikalischen Prozesse, oder beide, modifiziert<sup>1)</sup>. Der Zusatz des antagonistischen Salzes hemmt nun nach dieser Ansicht diese Erhöhung der Durchgängigkeit resp. verzögert die Geschwindigkeit, mit der diese Änderung eintritt. Eine physiologisch äquilibrierte Salzlösung ist also danach eine Lösung, in der die spezifische, für die Erhaltung des normalen Lebens der Zelle geeignete Permeabilität am längsten, ev. dauernd, erhalten bleibt.

Die beste Gelegenheit zur Untersuchung dieser antagonistischen Salzwirkungen bieten nun Organismen, die in hohem Grade von der Konzentration des umgebenden Mediums unabhängig sind, wie beispielsweise *Fundulus*. Eine Reihe von Versuchen an *Fundulus*, die ich in den letzten Jahren zum Teil im Verein mit H. Wasteneys ausgeführt habe, lassen sich leicht auf Grundlage der Theorie der antagonistischen Salzwirkungen verstehen<sup>2)</sup>. Von botanischer Seite ist diese Theorie von Osterhout angenommen worden, der neuerdings dieselbe durch eine sehr schöne Methode der elektrischen Widerstandsbestimmung an *Laminaria* gestützt hat<sup>3)</sup>.

Aber die Zahl der Beispiele, in denen eine direkte Stütze gewonnen war, ist noch immer sehr beschränkt. Die beste Stütze blieb nach wie vor die Tatsache, dass alle bisher gemachten Beobachtungen mit der Theorie übereinstimmen. Es war mir darum zu tun, mehr Beweismaterial zu finden und namentlich an dem Material, an dem die Theorie gewonnen war, nämlich am Ei von *Fundulus*, womöglich durch direkte Versuche den Nachweis zu führen, dass die antagonistische Salzwirkung in der Tat auf einer mehr oder weniger vollständigen Erhaltung der normalen spezifischen Durchgängigkeit der Zelle beruht. Dieser Nachweis ist mir nun auf Grund einer neuen Untersuchungsmethode am Ei von *Fundulus* gelungen.

Dabei gelang es aber auch, eine andere Frage in den Bereich der Untersuchung zu ziehen, nämlich ob Lipide oder Eiweisskörper

<sup>1)</sup> Arch. f. d. ges. Physiol. 107, 252, 1905. Vorlesungen über die Dynamik der Lebenserscheinungen, S. 81 u. ff. Leipzig 1906.

<sup>2)</sup> Science 34, 653, 1911.

<sup>3)</sup> Science 35, 112, 1912.

für die Erscheinung der spezifischen Permeabilität verantwortlich sind. Es werden Versuche angeführt, die darauf hinweisen, dass die Permeabilität der Membranen für Wasser und Salze durch die Eiweisskörper der Membran bestimmt ist.

Die folgenden Versuche sind alle am befruchteten Ei von *Fundulus* angestellt, und die neue Methode gründet sich auf den Nachweis der Erhöhung des spezifischen Gewichtes der Eier unter dem Einfluss hypertonischer Lösungen. Um diese Versuche zu verstehen, muss der Leser im Auge behalten, dass das Ei von einer derben Haut umgeben ist, die im folgenden als Eihaut oder als Eimembran bezeichnet wird. Innerhalb dieser Membran befindet sich der Dottersack mit dem Embryo; der Dottersack steht in keinem Zusammenhang mit der Eihaut, sondern ist frei innerhalb derselben beweglich. Normal ist die Eihaut straff gespannt, was darauf hinweist, dass das Ei prall mit einer Flüssigkeit gefüllt ist. Wenn man nun dem Ei durch Diffusion Wasser entzieht, so diffundiert zuerst das Wasser zwischen der Membran und dem Dottersack, wodurch die Eihaut oft, aber nicht immer, kollabiert oder Dellen erhält. Infolge der Erhöhung des osmotischen Druckes der Flüssigkeit zwischen Eihaut und Membran wird dann dem Dotter Wasser entzogen und derselbe schrumpft. Diese Schrumpfung führt zur Erhöhung des spezifischen Gewichtes des Eies. Auf diese Erhöhung des spezifischen Gewichtes des Eies gründet sich die neue, sehr bequeme Methode zur Untersuchung der Permeabilität der Eihaut von *Fundulus*.

Die Eier von *Fundulus* haben normalerweise ein spezifisches Gewicht, das erheblich höher ist als das von Seewasser; es liegt zwischen dem einer  $\frac{11}{8}$  und  $\frac{12}{8}$  m-NaCl-Lösung. Bringen wir die Eier in eine Lösung, deren spezifisches Gewicht über der eben erwähnten Grenze liegt, so werden sie so lange an der Oberfläche der Lösung schwimmen, als ihr spezifisches Gewicht niedriger ist als das der Lösung. Wäre nun die Durchgängigkeit der Eihaut unveränderlich, so sollte der Zeitpunkt des Sinkens abhängen 1. vom osmotischen Druck der umgebenden Lösung und 2. vom spezifischen Gewicht derselben. Je grösser der Unterschied des osmotischen Druckes der umgebenden Lösung und der Flüssigkeit im Ei, um so rascher muss das letztere Wasser verlieren; je höher das spezifische

Gewicht der umgebenden Lösung, um so länger wird es dauern, bis das Ei genug Wasser verloren hat, um zu sinken. Wir sehen aber, dass für die Geschwindigkeit des Sinkens an erster Stelle eine ganz andere Variable ausschlaggebend ist, nämlich die Natur der Lösung, in der die Eier sich befinden. Das weist darauf hin, dass die Permeabilität der Eihaut nicht konstant ist, sondern dass dieselbe durch die Lösung, in der sich das Ei befindet, modifiziert wird.

Im ersten Teil dieser Arbeit wollen wir nun zeigen, dass in Bezug auf die Geschwindigkeit des Sinkens ein prinzipieller Unterschied zwischen physiologisch äquilibrierten Lösungen und nicht äquilibrierten Lösungen besteht. In den letzteren sinken die Eier im allgemeinen rasch, in den ersteren sehr langsam, was wir auch auf Grund unserer Theorie der antagonistischen Salzwirkungen erwarten sollten.

Was nun die Methode der Versuche im einzelnen betrifft, so bestand sie darin, dass verschiedene Lösungen in Reagensgläser und je vier befruchtete Eier von *Fundulus* in je eine solche Lösung gebracht wurden; dann wurde die Zeit gemessen, während der die Eier an der Oberfläche der Lösung schwammen. Diese Lösungen mussten hypertonisch sein, d. h. ihre Konzentration musste höher sein als die des Seewassers oder einer  $\frac{3}{2}$  bis  $\frac{5}{8}$  m-NaCl-Lösung, denn sonst konnte das Ei kein Wasser verlieren. Andererseits durfte das spezifische Gewicht der Lösungen nicht so hoch sein, dass auch ein sehr starker Wasserverlust die Eier nicht zum Sinken bringen konnte. Es wurden deshalb Lösungen gewählt, die zwischen beiden Extremen lagen, beispielsweise 3 m Lösungen von NaCl (mit dem spezifischen Gewicht von 1,1303).

Es wurden nicht frisch befruchtete Eier benutzt, sondern Eier, die mindestens schon 3 Tage vorher befruchtet waren und einen Embryo mit Kreislauf besaßen. Ehe die Eier in die Reagensgläser gebracht wurden, wurden sie zweimal in destilliertem Wasser gewaschen.

## II. DAS SPEZIFISCHE GEWICHT DER FUNDULUSEIER.

Lösungen verschiedener Stoffe wurden hergestellt, um zu ermitteln, in welchen Lösungen die Eier sofort, d. h. in weniger als 5 Minuten zu Boden sinken. Um die Beobachtung zu erleichtern, wurden die Lösungen in Reagensgläser gebracht. Unter jeder Lösung der folgenden Tabelle ist die Dichtigkeit angegeben.

## Die Eier von Fundulus

sinken sofort in	schwimmen in
$\frac{11}{8}$ m-NaCl	$\frac{12}{8}$ m-NaCl
d = 1,0580	d = 1,0634
$\frac{10}{8}$ m-KCl	$\frac{12}{8}$ m-KCl
d = 1,0613	d = 1,0739
$\frac{4}{8}$ m-MgSO <sub>4</sub>	$\frac{6}{8}$ m-MgSO <sub>4</sub>
d = 1,0625	d = 1,0945
$\frac{6}{8}$ m-MgCl <sub>2</sub>	$\frac{8}{8}$ m-MgCl <sub>2</sub>
d = 1,0609	d = 1,0819
0,4 m-Rohrzucker	0,6 m-Rohrzucker
d = 1,05368	d = 1,08911

Das spezifische Gewicht der Funduluseier ist also ein wenig niedriger als 1,0634. Die Lösungen, in die die Eier gebracht wurden, um zu ermitteln, wie rasch ihr spezifisches Gewicht zunimmt, hatten ein erheblich höheres spezifisches Gewicht, nämlich 3 m-NaCl (d = 1,1303),  $\frac{10}{8}$  m-CaCl<sub>2</sub> (d = 1,1233) oder  $\frac{20}{8}$  m-CaCl<sub>2</sub> (d = 1,2641) usf.

III. DIE ENTWICKLUNG UND ÄNDERUNG DES SPEZIFISCHEN GEWICHTES DER FUNDULUSEIER IN LÖSUNGEN VON  
(NaCl + KCl + CaCl<sub>2</sub>) VERSCHIEDENER  
KONZENTRATION.

Die Mischungen von NaCl + KCl + CaCl<sub>2</sub> sind stets in dem Verhältnis hergestellt, in dem diese drei Salze im Seewasser enthalten sind, d. h. 100 Moleküle NaCl auf 2,2 Moleküle KCl und 1,5 Moleküle CaCl<sub>2</sub>. Eine solche Lösung ist eine physiologisch äquilibrierte Salzlösung, da die Giftwirkungen von KCl durch NaCl und CaCl<sub>2</sub> und die von NaCl durch KCl und CaCl<sub>2</sub> antagonisiert werden. Wir wollen nun zunächst zeigen, dass in Lösungen

dieser Art von hohem osmotischen Druck die spezifische Undurchgängigkeit der Eihaut von *Fundulus* gar nicht oder nur langsam geändert wird.

Wenn man eine solche Mischung verschiedener Konzentration von  $1/2$  bis  $2^{1/2}$  m herstellt und frisch befruchtete Eier in dieselben bringt, so findet man, dass dieselben sich bis zu Lösungen von  $15/8$  m zu Embryonen entwickeln<sup>1)</sup>. In Lösungen bis zu  $11/8$  m schlüpften auch die Fische aus, und zwar ein um so grösserer Prozentsatz, je geringer die Hypertonie der Lösung war. In Lösungen dieser drei Salze von  $12/8$  m und darüber schlüpfte kein Fisch mehr aus.

Wie ich vor vielen Jahren zeigte, sind die Fischeier in den ersten 24 Stunden empfindlicher gegen hohe Konzentrationen als später, und zwar dürfte das daran liegen, dass später die Membran oder Micropyle undurchgängiger wird. Wenn man 3 Tage alte Fischeier in eine  $2^{1/2}$  m-Lösung von  $\text{NaCl} + \text{KCl} + \text{CaCl}_2$  bringt, so bleiben dieselben eine Reihe von Tagen, manchmal 11 Tage und darüber, am Leben. Die Zirkulation bleibt während dieser Zeit im schönsten Gange.

Auch in destilliertem Wasser entwickeln sich die Eier vollständig und schlüpfen aus. Es wurden nun Eier, die sich 2 Tage lang in destilliertem Wasser befunden hatten, plötzlich in eine  $2^{1/2}$  m-Lösung von  $\text{NaCl} + \text{KCl} + \text{CaCl}_2$  gebracht. Die Embryonen lebten nahezu ebenso lange in dieser Lösung, wie die aus Seewasser übertragenen.

Diese und ähnliche Beobachtungen lassen sich unter folgenden zwei Voraussetzungen verstehen. 1. Bei Konzentrationen von Null bis etwa  $8/8$  m oder allenfalls  $10/8$  m- $\text{NaCl} + \text{KCl} + \text{CaCl}_2$  ist die Eihaut von *Fundulus* praktisch undurchgängig für Wasser und die in der Lösung ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ ) sowie die in dem Embryo enthaltenen Salze. 2. Bei höheren Konzentrationen wirkt diese Lösung langsam schädigend auf die Eihaut, wodurch dieselbe ihre Undurchgängigkeit für Wasser und Salze allmählich einbüsst, und zwar um so rascher, je höher die Konzentration der Lösung ist.

Um nun diese Ansicht zu prüfen, wurden die Eier in eine Reihe von Reagensgläsern mit folgenden Lösungen von  $\text{NaCl} + \text{KCl}$

<sup>1)</sup> Der osmotische Druck des Seewassers in Woods Hole liegt zwischen dem einer  $1/8$  und  $5/8$  m-Lösung von  $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ .



+  $\text{CaCl}_2$  gebracht:  $^{10}/_8$ ,  $^{11}/_8$ ,  $^{12}/_8$ ,  $^{13}/_8$ ,  $^{14}/_8$ ,  $^{15}/_8$ ,  $^{16}/_8$ ,  $^{18}/_8$  und  $^{20}/_8$  m. In jedes Reagensglas wurden dann je vier Funduluseier derselben Kultur gebracht, die neun Tage vorher befruchtet waren und alle einen lebenden Embryo hatten. In der  $^{10}/_8$  m-Lösung sanken die Eier sofort, in den übrigen blieben sie zunächst an der Oberfläche. Es war nun die Absicht, herauszufinden, wie lange die Eier oben schweben würden. Tabelle I gibt die Zahl der oben schwimmenden Eier nach verschiedenen Intervallen an.

TABELLE I.

Nach Tagen	Zahl der oben schwimmenden Eier in								
	$^{10}/_8$	$^{11}/_8$	$^{12}/_8$	$^{13}/_8$	$^{14}/_8$	$^{15}/_8$	$^{16}/_8$	$^{18}/_8$	$^{20}/_8$ m
	NaCl + KCl + $\text{CaCl}_2$								
1	0	1	2	4	4	3	4	3	3
2		0	1	3	4	3	4	3	3
3			0	0	3	2	4	3	3
4					1	1	4	1	3
5					0	0	1	0	3

Eine Untersuchung der Eier, die am Boden lagen, ergab eine Schrumpfung des Dotters, die um so beträchtlicher war, je höher die Konzentration der Lösung. Die Eier waren aber am 5. Tage praktisch alle am Leben und die Zirkulation noch im schönsten Gange. Es kann also eine Schrumpfung und ein Sinken der Eier stattfinden, ohne dass die Eier absterben. Diese Tatsache muss im Hinblick auf die später zu erwähnenden Beobachtungen hier betont werden.

Aus diesen Versuchen dürfen wir schliessen, dass in der Tat in hypertonischen Lösungen von NaCl + KCl +  $\text{CaCl}_2$  bis zu  $^{20}/_8$  m die spezifische Undurchgängigkeit der Eier von Fundulus eine Reihe von Tagen erhalten bleibt.

Es wurden nun Versuche mit Lösungen derselben Art, aber höherer Konzentrationen angestellt, nämlich mit 3, 4 und 5 m-Lösungen von NaCl + KCl +  $\text{CaCl}_2$  in dem Verhältnis, in dem diese Salze im Seewasser enthalten sind. In der 5 m-Lösung sanken zwei Eier bereits nach 2 Stunden und die beiden anderen kurz darauf. In allen Eiern in dieser Lösung war nach 2 Stunden der Dotter stark geschrumpft. Die Eier in 4 m- (NaCl + KCl +  $\text{CaCl}_2$ )

sanken erheblich langsamer, zwei Eier erst nach 24 Stunden. Die Eier in 3 m-(NaCl + KCl + CaCl<sub>2</sub>) lebten aber fast ebenso lange und schwammen nahezu ebenso lange an der Oberfläche wie in einer 2<sup>1</sup>/<sub>2</sub> m-(NaCl + KCl + CaCl<sub>2</sub>), d. h. 3 Tage oder länger.

Obwohl also eine 5 m-Lösung ein viel höheres spezifisches Gewicht besitzt als eine 3 m-Lösung, sinken die Eier doch in der 5 m-Lösung in wenig über 2 Stunden, während sie in der 3 m-Lösung 3 Tage oder länger an der Oberfläche schwimmen. Wir müssen daraus den Schluss ziehen, dass eine 5 m-Lösung von NaCl + KCl + CaCl<sub>2</sub> die Durchgängigkeit der Eimembran rasch erhöht und dass infolgedessen Wasser aus dem Ei austritt und vielleicht etwas Salz eintritt. Was bei dieser kritischen Konzentration rasch geschieht, geschieht bei niedrigen Konzentrationen derselben Mischung ebenfalls, aber langsamer.

Wir wollen nun zeigen, dass eine physiologisch nicht äquilibrierte Lösung die Durchgängigkeit der Membran schon bei relativ niedriger Konzentration rasch erhöht.

#### IV. DIE ÄNDERUNG DES SPEZIFISCHEN GEWICHTES DER EIER IN REINEN NaCl-LÖSUNG VERSCHIEDENER KONZENTRATION.

Die hier zu beschreibenden Versuche waren gleichzeitig und mit demselben Material angestellt, das auch zu den vorigen Versuchen benutzt wurde. Es wurden <sup>10</sup>/<sub>8</sub> bis <sup>20</sup>/<sub>8</sub> m-NaCl-Lösungen in verschiedene Reagensgläser und in jedes Reagensglas vier Funduluseier gebracht. In den <sup>10</sup>/<sub>8</sub> und <sup>11</sup>/<sub>8</sub> m-NaCl-Lösungen sanken die Eier sofort, weil natürlicherweise ihr spezifisches Gewicht etwas grösser ist als das einer <sup>11</sup>/<sub>8</sub> m-NaCl-Lösung.

TABELLE II.

Nach Minuten	Zahl der oben schwimmenden Eier in								20/8 m-NaCl
	10/8	11/8	12/8	13/8	14/8	15/8	16/8	18/8	
2	0	2	4	4	4	4	4	4	4
3		0	3	4	4	4	4	4	4
64			2	3	4	4	4	4	3
139			2	3	4	4	4	1	1
240			0	2	3	2	1	0	0
480				2	1	0	1	0	0

Am nächsten Morgen waren alle Eier unten.

Es wurde nun ein Versuch mit 5, 4 und 3 m-NaCl angestellt. In 2 Stunden waren die Eier in 5 und 4 m-NaCl alle zu Boden gesunken, die in 3 m-NaCl noch nicht, aber die Dotter waren bereits geschrumpft und die Embryonen waren tot (das Herz stand still). Nach ähnlichen Versuchen dürfen wir schliessen, dass die Eier kurz darauf zu Boden sanken, obwohl die Untersuchung hier ein paar Stunden unterbrochen wurde. Als die Eier in 3 m-NaCl wieder beobachtet wurden, lagen sie am Boden.

Wir sehen also, dass schon eine relativ niedrige Konzentration von NaCl die Durchgängigkeit der Eihaut sehr rasch erhöht. Wir sehen ferner, dass in den höheren Konzentrationen von NaCl die Eier trotz des höheren spezifischen Gewichtes der Lösung rascher sinken als in der niedrigen Konzentration. In Tabelle II sinken die Eier in einer  $\frac{20}{8}$  m-NaCl-Lösung alle in weniger als 4 Stunden, während in der  $\frac{13}{8}$  m-NaCl-Lösung noch zwei Eier nach 8 Stunden schwimmen. Nirgends aber schwimmt ein Ei 24 Stunden lang. Dem Sinken ging wie immer ein Schrumpfen des Dottersackes voraus. Es fand also sicher ein Austritt von Wasser aus der Eihaut statt. Während aber in einer  $\frac{20}{8}$  m-Lösung von NaCl + KCl +  $\text{CaCl}_2$  der zum Sinken erforderliche Austritt von Wasser in etwa 3 bis 5 Tagen stattfindet, findet derselbe in der  $\frac{20}{8}$  m-NaCl-Lösung in ebenso viel Stunden oder noch weniger statt. Dieser Unterschied in der Geschwindigkeit des Wasseraustrittes in der physiologisch äquilibrierten Lösung (NaCl + KCl +  $\text{CaCl}_2$ ) und in der nicht äquilibrierten Lösung (NaCl) kann nur darauf beruhen, dass die reine NaCl-Lösung die natürlicherweise für Wasser fast undurchgängige Eimembran für Wasser rasch durchgängig macht, während diese Erhöhung der Durchgängigkeit in der physiologisch äquilibrierten Lösung nur langsam eintritt.

Während nun in den Lösungen von NaCl + KCl +  $\text{CaCl}_2$  der Embryo meist noch am Leben ist, wenn die Eier schon sinken, ist in den reinen NaCl-Lösungen das umgekehrte der Fall; die Embryonen sterben meist schon, ehe der Dotter so weit geschrumpft ist, dass das Ei sinkt. Dieser Unterschied findet m. E. seine Erklärung darin, dass nicht nur die Durchgängigkeit der Membran für Wasser erhöht wird, sondern, wenn auch in geringerem Grade, diejenige für Salze. Wenn nun die reine NaCl-Lösung in das Ei eindringt, so

tötet das den Embryo natürlich rascher, als wenn  $\text{NaCl} + \text{KCl} + \text{CaCl}_2$  eintritt.

Wir verstehen auch aus diesen Versuchen, warum allgemein Lösungen hoher Konzentration Cytolyse der Zellen verursachen. Über eine gewisse Konzentration hinaus zerstört eben jede Lösung die spezifische Permeabilität einer Membran, und zwar um so rascher, je höher die Konzentration.

#### V. DIE ÄNDERUNG DES SPEZIFISCHEN GEWICHTES DER EIER VON FUNDULUS IN LÖSUNGEN VON $\text{CaCl}_2$ .

Die Versuche mit  $\text{CaCl}_2$  gaben ein ganz unerwartetes Resultat: Die befruchteten Eier fingen in den Lösungen von höherem spezifischen Gewicht schon in etwa  $\frac{1}{2}$  Stunde an zu sinken. Eier, die vor drei Tagen befruchtet waren, wurden in  $\frac{6}{8}$ ,  $\frac{8}{8}$ ,  $\frac{10}{8}$ ,  $\frac{12}{8}$ ,  $\frac{14}{8}$ ,  $\frac{16}{8}$ ,  $\frac{18}{8}$  und  $\frac{20}{8}$  m-Lösungen von  $\text{CaCl}_2$  gebracht. In einer  $\frac{m}{2}$ - $\text{CaCl}_2$ -Lösung sinken alle Eier sofort; in einer  $\frac{6}{8}$  m-Lösung sinken einige, während andere schwimmen; in den stärker konzentrierten Lösungen schwammen sie an der Oberfläche. Nach 25 Minuten beginnen die ersten Eier zu sinken<sup>1)</sup>, und 6 Minuten später sind die Eier in allen Gefässen im Sinken oder am Boden. Obwohl der Vorgang sehr rasch verläuft, kann man doch feststellen, dass in den niedrigen Konzentrationen von  $\text{CaCl}_2$ , nämlich  $\frac{8}{8}$ ,  $\frac{10}{8}$  und  $\frac{12}{8}$  m die Eier ein wenig später zu sinken beginnen als in den stärker konzentrierten Lösungen.

Verfolgt man nun das Verhalten eines 8 Tage alten Embryos unter dem Mikroskop, während derselbe einer  $\frac{10}{8}$  m- $\text{CaCl}_2$ -Lösung ausgesetzt ist, so beobachtet man, dass sich fast sofort Dellen in der Einmembran bilden (was auf einen Flüssigkeitsaustritt hinweist); diese Dellen können aber später wieder verschwinden. Der Embryo macht plötzlich sehr lebhaft Bewegungen, die auf ein Eindringen von  $\text{CaCl}_2$  in das Ei hinweisen. In etwa 10 bis 15 Minuten hört schon die Zirkulation und bald auch der Herzschlag auf. Inzwischen hat auch die Schrumpfung des Dotters begonnen, die nun

<sup>1)</sup> Es sei hier bemerkt, dass der Zeitpunkt, in dem das Ei ein grösseres spezifisches Gewicht erreicht als die Lösung, in der es sich befindet, oft dadurch der Aufmerksamkeit entgeht, dass die Oberflächenspannung das Ei am Sinken hindert. Deshalb ist es nötig, von Zeit zu Zeit die Reagensgläser leicht zu schütteln.

rasch weiterschreitet. Wir müssen daraus schliessen, dass hyper-tonische Lösungen von  $\text{CaCl}_2$  die Eihaut in wenigen Minuten durchgängig machen, dass infolgedessen Wasser aus dem Ei tritt, wodurch der Dotter zum Schrumpfen kommt, und dass etwas  $\text{CaCl}_2$  in das Ei tritt und den Embryo rasch tötet. Die Erfahrung, dass  $\text{CaCl}_2$  schon in so niedriger Konzentration die Durchlässigkeit der Eihaut so rasch erhöht, ist überraschend im Hinblick auf die Erfahrungen der Botaniker. Ich vermute aber, dass die Aussagen der letzteren sich auf  $\text{CaCl}_2$ -Lösungen geringerer Konzentration beziehen. Ich habe ja schon in einer früheren Arbeit darauf hingewiesen, dass  $\text{CaCl}_2$  in niedrigerer Konzentration die entgegengesetzte Wirkung hat, welche ihm in höheren Konzentrationen zukommt; in niedrigen Konzentrationen verringert es die Durchgängigkeit, während es in höheren Konzentrationen dieselbe erhöht<sup>1)</sup>.

VI. BEWEIS, DASS DIE ANTAGONISTISCHE SALZWIRKUNG AUF DER  
ERHALTUNG DER SPEZIFISCHEN DURCHLÄSSIGKEIT DER  
EIIHAUT BERUHT.

In früheren Arbeiten habe ich gezeigt, dass, wenn wir frisch befruchtete Eier von *Fundulus* in eine  $\frac{5}{8}$  m-NaCl-Lösung bringen, das Ei so rasch getötet wird, dass kein Embryo gebildet wird; dass, wenn wir aber eine sehr kleine, aber bestimmte Menge eines Erdalkalisalzes zusetzen, alle Eier Embryonen bilden. Wir wollen nun zeigen, dass der Zusatz eines solchen Salzes zu einer NaCl-Lösung die Erhöhung der Durchgängigkeit der Eihaut hemmt, die durch das NaCl allein herbeigeführt wird.

Wir stellen folgenden Versuch an, den ich vielen Besuchern gezeigt habe und der ein nie versagender Demonstrationsversuch ist. Drei Lösungen werden in drei Reagensgläser *a*, *b* und *c* verteilt. In *a* befindet sich eine 3 m-NaCl-Lösung, in *b* eine  $\frac{10}{8}$  m- $\text{CaCl}_2$ -Lösung, und in *c* eine Mischung von 48 ccm 3 m-NaCl + 2 ccm  $\frac{10}{8}$  m- $\text{CaCl}_2$ . In jedes Reagensglas kommen 4 Eier von *Fundulus*, welche etwa 3 bis 10 Tage vorher befruchtet waren. Zunächst schwimmen die Eier in allen 3 Gefässen an der Oberfläche. In 30 Minuten sind alle 4 Eier in *b* ( $\frac{10}{8}$  m- $\text{CaCl}_2$ ) am Boden; die Dotter der Eier sind stark geschrumpft, die Embryonen tot. Nach weni-

<sup>1)</sup> Diese Zeitschr. 39, 194, 1912.

gen Stunden (etwa 3 in der Regel) sind in *a* (3 m-NaCl) alle 4 Embryonen tot und die Dotter geschrumpft, und die Eier sind am Boden. In *c* (48 ccm 3 m-NaCl + 2 ccm  $^{10}/_8$  m-CaCl<sub>2</sub>) sind alle 4 Eier noch nach 3 bis 4 Tagen am Leben und an der Oberfläche. Nach 3 Tagen ist oft noch keine Schrumpfung dieser Eier bemerklich und die Herztätigkeit und die Zirkulation sind noch im Gange. Also bei den Eiern, die sich in der physiologisch äquilibrierten Lösung (48 ccm 3 m-NaCl + 2 ccm  $^{10}/_8$  m-CaCl<sub>2</sub>) befinden, tritt die Erhöhung des spezifischen Gewichts, die zum Sinken führt, erst nach etwa 3 bis 4 oder allenfalls 5 Tagen ein, während sie in der  $^{10}/_8$  m-CaCl<sub>2</sub>-Lösung nach 30 Minuten, in der 3 m-NaCl-Lösung in etwa 90 bis 180 Minuten eintritt.

Wir sehen also, dass in diesem Versuch sowohl die  $^{10}/_8$  m-CaCl<sub>2</sub>-Lösung als auch die 3 m-NaCl-Lösung jede für sich den Embryo rasch tötet und den Dottersack in weniger als 1 resp. 3 Stunden zum Schrumpfen und damit das Ei zum Sinken veranlasst, während die Mischung beider Lösungen im Verhältnis von 25 ccm 3 m-NaCl + 1 ccm  $^{10}/_8$  m-CaCl<sub>2</sub> die Embryonen am Leben erhält, und das Schrumpfen des Dottersackes und damit das Sinken der Eier verhindert.

Hier ist nun anscheinend eine Schwierigkeit vorhanden, nämlich, dass eine  $^{10}/_8$  m-CaCl<sub>2</sub>-Lösung allein die Durchlässigkeit rascher erhöht als eine 3 m-NaCl-Lösung, und dass trotzdem der Zusatz von einer kleinen Menge von CaCl<sub>2</sub> zu NaCl die Durchgängigkeitserhöhung hindert, welche das NaCl allein hervorrufen würde. Ich glaube, dass dieser scheinbare Widerspruch seine Erledigung findet in der Tatsache, auf die ich schon früher hingewiesen habe, dass CaCl<sub>2</sub> in niedriger Konzentration eine schützende Wirkung ausübt, während es in höherer Konzentration die Membran schädigt. Wenn wir 1 ccm  $^{10}/_8$  m-CaCl<sub>2</sub> zu 24 ccm 3 m-NaCl zufügen, so ist die Konzentration des CaCl<sub>2</sub> in der Mischung  $^{m}/_{20}$ . In dieser Konzentration hat das CaCl<sub>2</sub> aber nur eine schützende Wirkung auf die Membran. Die Tatsache, dass die schädliche Wirkung einer Salzlösung auf die Membran eine Funktion der Konzentration ist, wird ja in all diesen Versuchen klar.

Wir wollen nun zeigen, dass auch die Salze der anderen Erdalkalimetalle, SrCl<sub>2</sub>, MgCl<sub>2</sub> und BaCl<sub>2</sub>, die das NaCl zu entgiften im-

stande sind, auch die Durchlässigkeitserhöhung der Eihaut durch NaCl hemmen.

Es wurden je 4 Eier (9 Tage nach der Befruchtung) in  $\text{SrCl}_2$  verschiedener Konzentration gebracht. In einer  $\frac{2}{8}$  m- $\text{SrCl}_2$ -Lösung sinken die Eier sofort, in Lösungen von  $\frac{4}{8}$  m und darüber schwimmen dieselben an der Oberfläche.

TABELLE III.

Nach	Zahl der oben schwimmenden Eier in								20/8 m- $\text{SrCl}_2$
	4/8	6/8	8/8	10/8	12/8	14/8	16/8	18/8	
1 Stunde	4	4	4	4	2	0	0	1	4
3 Stunden	4	4	0	0	0			0	2
22 "	4	0							0

Die Eier beginnen in den Lösungen höherer Konzentration rascher zu sinken als in den niedrigeren Konzentrationen, was wohl darauf hinweist, dass die Verringerung der Durchlässigkeit der Eihaut um so rascher eintritt, je höher die Konzentration ist. Dass die  $\frac{18}{8}$  und  $\frac{20}{8}$  m-Lösungen eine scheinbare Ausnahme bilden, ist nur durch das hohe spezifische Gewicht dieser Lösungen bedingt. Die Eier waren nämlich stark geschrumpft. Es ist beachtenswert, dass eine  $\frac{4}{8}$  m-Lösungen von  $\text{SrCl}_2$  das Ei weniger rasch tötet als eine  $\frac{4}{8}$  m- $\text{CaCl}_2$ -Lösung. Es wurde nun untersucht, ob der Zusatz von  $\text{SrCl}_2$  zu einer 3 m-NaCl-Lösung das Sinken der Eier in dieser Lösung zu hemmen imstande sei. Zu dem Zwecke wurden in eine Reihe von Reagensgläsern die in der folgenden Tabelle angegebenen Mischungen von 3 m-NaCl und  $\frac{10}{8}$  m- $\text{SrCl}_2$  gebracht und zu jeder Lösung 4 Eier zugesetzt.

TABELLE IV.

Nach		Zahl der oben schwimmenden Eier in 50 ccm 3 m-NaCl +						
		0	0,1	0,2	0,4	0,8	1,6	3,2
		ccm $\frac{10}{8}$ m- $\text{SrCl}_2$						
2 Stunden		2	3	3	4	4	4	4
4 " 50 Min.		0	0	3	3	4	4	4
22 "				0	0	1	3	4
2 Tagen						0	2	4
3 "							2	4
4 "							0	
5 "								

Man sieht also, dass der Zusatz von 3,2 ccm  $^{10}/_8$  m-SrCl<sub>2</sub> zu 50 ccm 3 m-NaCl die schädigende Wirkung der letzteren Lösung auf die Eihaut fast in demselben Masse vermindert, wie der Zusatz von CaCl<sub>2</sub>. Wie im Falle von CaCl<sub>2</sub> besteht auch hier die schützende Wirkung von SrCl<sub>2</sub> darin, dass seine Gegenwart die Erhöhung der Durchlässigkeit der Eihaut für Wasser und Salze, die durch 3 m-NaCl rasch herbeigeführt würde, hemmt.

In den früheren Versuchen über die Hemmung der Giftwirkung einer reinen NaCl-Lösung auf Fische durch die Salze zweiwertiger Kationen zeigte es sich, dass die entgiftende Wirkung von CaCl<sub>2</sub> und SrCl<sub>2</sub> nahezu gleich war, dass aber die von MgCl<sub>2</sub> und besonders von BaCl<sub>2</sub> erheblich geringer war. Es wurde untersucht ob sich ein ähnlicher Unterschied auch in den Versuchen über die Erhöhung des spezifischen Gewichtes der Eier nachweisen liesse.

Bringt man die Eier in MgCl<sub>2</sub>-Lösungen von verschiedener Konzentration, so benehmen sie sich ähnlich wie in den CaCl<sub>2</sub>-Lösungen. Je vier Eier wurden in  $^{2}/_8$ ,  $^{4}/_8$ ,  $^{6}/_8$ ,  $^{8}/_8$ ,  $^{10}/_8$ ,  $^{12}/_8$ ,  $^{14}/_8$ ,  $^{16}/_8$  und  $^{20}/_8$  m-MgCl<sub>2</sub> gebracht. In  $^{2}/_8$  und  $^{4}/_8$  m-MgCl<sub>2</sub> sinken die Eier sofort und rasch, da ihr spezifisches Gewicht grösser ist als das der Lösung. In  $^{6}/_8$  m-MgCl<sub>2</sub> sinken sie etwas langsamer; das spezifische Gewicht dieser Lösung ist 1,0609, und das ist nur wenig geringer als das spezifische Gewicht der Eier. In den Lösungen höherer Konzentration schwimmen sie an der Oberfläche. Nach etwa 30 Minuten fangen die Eier an in allen Lösungen zu Boden zu sinken, zuletzt in der  $^{10}/_8$  m und  $^{8}/_8$  m-Lösung, in denen sie etwas länger als 30 Minuten an der Oberfläche bleiben können. Dem Sinken geht in diesem Falle wie immer in Lösungen von höherem spezifischen Gewicht eine Schrumpfung des Dotters voraus; ausserdem starben die Embryonen in diesem Versuche auch rasch. MgBr<sub>2</sub> wirkt ähnlich wie MgCl<sub>2</sub>. In einer Versuchsreihe wurden zu 50 ccm 3 m-NaCl verschiedene Mengen einer  $^{10}/_8$  m-MgBr<sub>2</sub>-Lösung zugesetzt und je vier Eier in ein Reagensglas gebracht.

Während also in den 3 m-NaCl-Lösungen die Eier schon in den ersten 3 Stunden oder weniger sanken, genügte der Zusatz von 3,2 ccm  $^{10}/_8$  m-MgBr<sub>2</sub>, einen Teil der Eier 6 bis 22 Stunden vor dem Sinken zu bewahren. Die Schutzwirkung von MgBr<sub>2</sub> ist also, wie zu erwarten, geringer als die von CaCl<sub>2</sub> und SrCl<sub>2</sub>. Bringt man



TABELLE V.

Nach	Zahl der oben schwimmenden Eier in 50 ccm 3 m-NaCl +								12,8 ccm 10/8-MgBr <sub>2</sub>
	0	0,1	0,2	0,4	0,8	1,6	3,2	6,4	
70 Minuten	1	4	4	4	4	4	4	4	4
195 "	0	0	0	0	4	4	4	4	4
6 Stunden					0	0	2	4	1
23 "							0	2	1
2 Tagen								0	0

Fundulus-Eier in Lösungen von BaCl<sub>2</sub> mit höherem spezifischen Gewicht als das der Eier, so fangen nach einiger Zeit die Eier an zu sinken. Lösungen von 0,4 m-BaCl<sub>2</sub> und darüber haben ein höheres spezifisches Gewicht als die Eier. Je vier Eier wurden in die BaCl<sub>2</sub>-Lösungen der Tabelle VI gebracht.

TABELLE VI.

Nach Minuten	Zahl der oben schwimmenden Eier in						0,9 m-BaCl <sub>2</sub>
	0,4	0,5	0,6	0,7	0,8		
60	4	4	4	3	2		1
94	4	4	3	1	1		0
150	3	2	1	0	0		
235	1	1	0				
355	1	0					

Der Versuch wurde ausführlich mitgeteilt, weil derselbe besser als die bisherigen Versuche die Tatsache zum Ausdruck bringt, dass die höher konzentrierten Lösungen die Erhöhung der Permeabilität der Membran schneller bewirken als Lösungen niedriger Konzentration. Darauf beruht es wohl, dass für dieses Ei für jede Lösung eine Grenzkonzentration gefunden werden kann, unterhalb deren das Fundulusei beliebig lange lebt, weil die Lösung unterhalb dieser Konzentration nicht imstande ist, die Durchgängigkeit der Membran über den kritischen Wert zu erhöhen.

Dass nun der Zusatz von etwas BaCl<sub>2</sub> auch das Sinken der Eier in einer 3 m-NaCl-Lösung hemmen kann, wird im folgenden Versuch gezeigt. Wie immer wurden je vier Eier in ein Reagensglas mit der Lösung gebracht. Die Eier waren vor 7 Tagen befruchtet.

Der Zusatz von 4,0 m-BaCl<sub>2</sub> zu 100 ccm 3 m-NaCl verzögert die Erhöhung der Durchlässigkeit der Eimembran, die die 3 m-NaCl-Lösung an sich bewirkt, ganz erheblich.

Diese Versuche beweisen, dass die Entgiftung einer reinen NaCl-Lösung durch eine kleine Menge eines Erdalkalisalzes beim Fundulusei in der Tat in erster Linie darauf beruht, dass das Erdalkalisalz die Erhöhung der Permeabilität der Eihaut hemmt, die durch die NaCl-Lösung hervorgebracht wird.

TABELLE VII.

Nach	Zahl der oben schwimmenden Eier in 100 ccm 3 m-NaCl +									
	0	0,1	0,2	0,4	1,2	1,6	4,0	8,0	16,0	32,0 m-BaCl <sub>2</sub>
180 Minuten	0	0	4	4	4	4	4	4	3	3
290 "			0	4	4	3	4	4	1	1
6½ Stunden				1	1	2	4	1	1	0
10 "				0	1	0	1	0	0	

Damals wurde gefunden, dass nicht nur die Salze von Mg, Ca, Sr und Ba, die giftige Wirkung einer  $\frac{5}{8}$  m-NaCl-Lösung oder einer Lösung eines Salzes mit einwertigem Metall auf das frisch befruchtete Ei aufheben, sondern auch die Salze von Zn und Pb, u. a. Es wurde nun der Versuch gemacht, ob der Zusatz von etwas ZnSO<sub>4</sub> auch das Sinken der Eier in einer Lösung von 3 m-NaCl verzögern würde. Das Gegenteil war aber der Fall, der Zusatz von ZnSO<sub>4</sub> beschleunigte das Sinken der Eier in dieser Lösung.

Da die Lösung von ZnSO<sub>4</sub> sauer ist, so lag es nahe, dieses Verhalten der Lösung der Säure zuzuschreiben. Es wurden deshalb einer 3 m-NaCl-Lösung verschiedene Mengen  $\frac{m}{10}$ -HCl zugesetzt. Es stellte sich heraus, dass der Zusatz selbst sehr kleiner Mengen von Säure zu der 3 m-NaCl-Lösung das Sinken der Eier in der Lösung beschleunigte. Da wir später eingehend die Wirkung von Säure auf die Durchgängigkeit der Eihaut besprechen, so wollen wir von der Mitteilung von Zahlenbeispielen absehen.

Es entsteht aber dann die Frage, wie es zu erklären ist, dass in den früheren Versuchen der Zusatz von etwas ZnSO<sub>4</sub> die schädliche Wirkung der reinen NaCl-Lösung aufhob. Das dürfte wohl daran liegen, dass wir damals nicht mit 3 m-NaCl, sondern mit  $\frac{5}{8}$  m-NaCl arbeiteten. Wenn wir nun dieser letzteren NaCl-Lösung ZnSO<sub>4</sub> zusetzten, so hatten wir in Wirklichkeit folgendes System:  $\frac{5}{8}$  m-NaCl —  $\frac{m}{80}$  ZnSO<sub>4</sub> — niedrige Konzentration HCl. Die  $\frac{5}{8}$  m-NaCl-Lösung wirkte antagonistisch auf die Säure; und Zn

wirkte antagonistisch auf  $\frac{5}{8}$ -m-NaCl; und möglicherweise wirkte NaCl entgiftend auf  $\text{ZnSO}_4$ . Nun fanden Wasteneys und ich, dass Salzlösungen nur so lange Fundulus gegen Säure schützen, als die Konzentration der Salzlösung die Grenze der Isotonie nicht übersteigt. Steigt die Konzentration der Salzlösung über  $\frac{m}{2}$ , so nimmt die schützende Wirkung derselben gegen Säure rasch ab<sup>1)</sup>. In meiner neuen Versuchsreihe war die Konzentration der NaCl-Lösung 3 m. Eine solche Lösung wirkt nicht länger antagonistisch auf Säure, sondern, im Gegenteil, durch Säurezusatz wird die schädliche Wirkung der 3 m-NaCl-Lösung auf die Eihaut beschleunigt.

Es wurden weiter Versuche angestellt, ob es auch gelingt, das Sinken der Eier in einer 4 m-NaCl-Lösung durch Zusatz von Salzen mit zweiwertigem Metall zu hemmen. Das war wohl der Fall, aber es gelang, wie zu erwarten, nicht so gut wie im Falle einer 3 m-Lösung von NaCl. So sanken in einem Versuch in 50 ccm 4 m-NaCl die Eier in weniger als 2 Stunden, während dieselben in 50 ccm 4 m-NaCl + 1 ccm  $2\frac{1}{2}$  m- $\text{CaCl}_2$  noch nach 5 Stunden alle oben waren. Nach 17 Stunden, und wahrscheinlich schon früher, waren sie aber auch alle am Boden.

Wie NaCl, verhält sich auch LiCl und KCl. Es würde zu weit führen, alle diese Versuche hier anzuführen, aber ein KCl-Versuch möge erwähnt werden, weil er das Eindringen des Salzes in schöner Weise zeigt. Je vier Eier wurden in folgende Lösungen gebracht: 50 ccm  $\frac{12}{8}$  m-KCl + 0, 0,8, 1,6, 3,2 ccm  $\frac{10}{8}$ - $\text{CaCl}_2$ . In allen stockte der Herzschlag in weniger als 30 Minuten, ein Beweis, dass etwas KCl eingedrungen war. Aber während am nächsten Morgen die Eier in  $\frac{12}{8}$  m-KCl alle am Boden waren, befanden sich in den calciumhaltigen  $\frac{12}{8}$  m-KCl-Lösungen noch je zwei Eier an der Oberfläche. Der Versuch beweist, dass die Wasserabgabe aus dem Ei durch die Gegenwart von Ca mehr verlangsamt wird, als das Eindringen des KCl in das Ei.

Wir besitzen mithin in den neuen Versuchen über die Änderung des spezifischen Gewichtes der Eier einen direkten Beweis, dass die Aufhebung der Giftwirkung einer Lösung eines Salzes mit einwertigem Metall (z. B. NaCl) durch Zusatz einer kleinen Menge eines

<sup>1)</sup> Diese Zeitschr. 39, 167, 1912.

Salzes mit zweiwertigem Metall (z. B.  $\text{CaCl}_2$ ) darauf beruht, dass das  $\text{CaCl}_2$  die Erhöhung der Durchlässigkeit der Eihaut hemmt, die das  $\text{NaCl}$  in hinreichender Konzentration hervorruft. In einer Mischung von  $\text{NaCl}$  und  $\text{CaCl}_2$  (in richtigem Verhältnis) bleibt die Membran für Wasser und Salze relativ lange undurchgängig.

#### VII. ÜBER EINE VERBESSERTE METHODE ZUR MESSUNG DER ERHÖHUNG DER DURCHLÄSSIGKEIT.

Es ist für die weitere Ausarbeitung dieses Gebietes sehr wichtig, dass sich die Erhöhung der Durchlässigkeit der Membran durch eine Lösung messend verfolgen lässt. Zu dem Zweck lässt sich die folgende Methode benutzen. Man nimmt Eier einer Kultur und setzt sie verschieden lange der Einwirkung einer Lösung aus, welche die Durchgängigkeit der Membran erhöht, z. B.  $\frac{10}{8}$  m- $\text{CaCl}_2$ . Dann werden die Eier immer in dieselbe hypertonische äquilibrierte Lösung gebracht, in der sie normalerweise lange schwimmen. Es wird dann gemessen, wie rasch sie in dieser Lösung zu Boden sinken. Wir benutzten als eine solche physiologisch äquilibrierte Probelösung eine Mischung von 50 ccm 3 m- $\text{NaCl}$  + 1 ccm  $2\frac{1}{2}$  m- $\text{CaCl}_2$ . In einer solchen Lösung schwimmen normale Eier etwa 3 Tage lang. (Diese Lösung soll nun kurz im folgenden als die Probelösung bezeichnet werden.)

Eier, die 7 Tage vorher befruchtet worden waren, wurden zweimal in destilliertem Wasser gewaschen und dann in  $\frac{10}{8}$  m- $\text{CaCl}_2$  gebracht. Eine Portion der Eier wurde unter dem Mikroskop in der Lösung beobachtet. Nach 12 Minuten stand bei der Hälfte der Embryonen bereits das Herz still und 2 Minuten später stand bei allen das Herz still. Um diese Zeit war die Schrumpfung des Dotters noch sehr gering, dieselbe schritt aber rasch vor. Nach 23 Minuten begannen die Eier zu Boden zu sinken. 1, 2, 3, 4,  $7\frac{1}{2}$  und 10 Minuten, nachdem die Eier in die  $\frac{10}{8}$  m-Lösung von  $\text{CaCl}_2$  übertragen waren, wurden je 4 derselben in die Lösung von 50 ccm 3 m- $\text{NaCl}$  + 1 ccm  $2\frac{1}{2}$  m- $\text{CaCl}_2$  übertragen. Vier nicht mit  $\text{CaCl}_2$  behandelte Eier wurden zur Kontrolle ebenfalls in diese Lösung gebracht. Das Resultat gibt Tabelle VIII.

TABELLE VIII.

Nach	Zahl der in der Probeflösung schwimmenden Eier. Vorbehandlung mit 10/8 m-CaCl <sub>2</sub>						
	0	1	2	3	4	7½	10 Min.
85 Minuten	4	4	4	1	0	0	0
4½ Stunden	4	3	2	0			
17 Stunden	4	2	0				

Dieser Versuch zeigt, dass bereits ein Aufenthalt der Eier von wenigen Minuten in  $10/8$  m-CaCl<sub>2</sub> die normale Undurchgängigkeit ihrer Membranen fast völlig beseitigt. Die Eier schwimmen in einer Lösung von 50 ccm 3 m-NaCl + 1 ccm  $2^{1/2}$  m-CaCl<sub>2</sub> mehrere Tage, weil sie relativ undurchgängig für Wasser und Salze sind und diese Lösung ihre Undurchgängigkeit nur sehr langsam verringert. Bringt man sie aber nur 4 Minuten in eine  $10/8$  m-NaCl-Lösung, so sinken sie hinterher in einer 3 m-NaCl + CaCl<sub>2</sub>-Lösung schon in weniger als 85 Minuten alle zu Boden. Ein Aufenthalt von 4 Minuten in der CaCl<sub>2</sub>-Lösung bringt aber keine äusserlich wahrnehmbare Veränderung bei den Eiern hervor, und sie entwickeln sich normal, wenn man sie nach dieser Zeit in Seewasser zurückbringt. Selbst ein Aufenthalt von 2 oder sogar 1 Minute in der reinen  $10/8$  m-CaCl-Lösung erhöht die Durchgängigkeit der Eier schon merklich.

In einer 3 m-NaCl-Lösung sinken die Eier ebenfalls in  $1^{1/2}$  bis 3 Stunden. Nach unserer Annahme muss dem Sinken der Eier eine Erhöhung der Durchgängigkeit ihrer Membran vorausgehen. Da

TABELLE IX.

Nach	Zahl der in der Probeflösung schwimmenden Eier. Vorbehandlung in 3 m-NaCl von						
	0	11	21	41	62	74	88 Min.
2 Stunden	4	4	4	4	3	3	2
5 " 20 Min.	4	4	4	3	0	2	1
7 "	4	3	3	2	0	2	0
24 "	4	2	1	1		1	

aber das Sinken der Eier in einer 3 m-NaCl-Lösung langsamer erfolgt als in einer  $10/8$  m-CaCl<sub>2</sub>-Lösung, so muss auch die Erhöhung der Durchgängigkeit langsamer erfolgen. Eier, die vorher

in destilliertem Wasser gewaschen waren, wurden in eine 3 m-Lösung von NaCl gebracht und nach verschiedenen Intervallen wurden je 4 Eier von hier in Reagensgläser mit 50 ccm 3 m-NaCl + 1 ccm  $2\frac{1}{2}$  m-CaCl<sub>2</sub> übertragen. Tabelle IX gibt das Resultat.

Man sieht, dass schon nach 60 Minuten die Durchgängigkeit der Eihaut in einer 3 m-NaCl-Lösung so stark erhöht wird, dass die Eier in einer Lösung von 50 ccm 3 m-NaCl + 1 cmm  $2\frac{1}{2}$  m-CaCl<sub>2</sub>, in der sie 3 Tage lang oben schwimmen sollten, schon nach 5 Stunden zu Boden sinken.

Als weiteres Beispiel möge die Wirkung von Kaliumoleat dienen. Eier wurden in  $\frac{m}{100}$ -Kaliumoleat gebracht. Nach 10 Minuten wurden die Embryonen sehr unruhig, ein Beweis für das Eindringen der Seife. Nach etwa 1 Stunde traten Störungen der Zirkulation auf. Nach verschiedenen Intervallen wurden Eier aus der Seifenlösung genommen, zweimal in H<sub>2</sub>O gewaschen und dann in 50 ccm 3 m-NaCl + 1 ccm  $2\frac{1}{2}$  m-CaCl<sub>2</sub> übertragen. Die nach 36 Minuten aus der Seifenlösung genommenen Eier besaßen bereits eine so grosse Durchlässigkeit der Eihaut, dass dieselben schon nach 2 Stunden in der Probelösung zu Boden sanken. Die nach 51 Minuten herausgenommenen Eier sanken schon erheblich früher zu Boden. Ebenso liess sich die Erhöhung der Durchlässigkeit durch Natriumoxalat nachweisen.

Ich glaube, dass diese und ähnliche Versuche den Beweis liefern, dass die Eihaut von Fundulus im Seewasser als undurchgängig für Wasser und Salze angesehen werden darf; dass Lösungen von nur einem Salz die Membran durchgängig für Wasser und Salze machen und dass die Geschwindigkeit, mit der die Erhöhung der Durchgängigkeit eintritt, eine Funktion der Natur und Konzentration der Lösung ist. Damit ist auch das Wesen der antagonis-tischen Salzlösung aufgeklärt: der Zusatz von 1 ccm  $2\frac{1}{2}$  m-CaCl<sub>2</sub> verhindert die rasche Erhöhung der Durchgängigkeit der Eihaut, welche eine reine 3 m-NaCl-Lösung hervorbringen würde.

VIII. DIE BEDEUTUNG DER PROTEINE FÜR DIE DURCHGÄNGIGKEIT  
DER EIHAUT FÜR WASSER UND SALZE.

Die weit verbreitete Annahme, dass die Lipide der Zellmembran die Aufnahme und Abgabe von Stoffen durch die Zelle bestimmen, stösst auf die unüberwindliche Schwierigkeit, dass der Stoffaustausch der Zelle auf Wasser und wasserlösliche und nicht auf lipoidlösliche Stoffe eingestellt ist. Um dieser Schwierigkeit zu entgehen, haben Overton u. a. auf die Möglichkeit hingewiesen, dass für die Absorption von Wasser, Salzen und anderen lipoidunlöslichen Stoffen nicht die Diffusionskräfte, sondern ein noch unbekannter Mechanismus in Betracht komme. Ich habe schon früher darauf hingewiesen, dass es doch näher liegt, die Möglichkeit in Betracht zu ziehen, dass die Absorption lipoidunlöslicher Stoffe überhaupt nichts mit den Lipiden zu tun hat, sondern dass hierfür andere Stoffe, z. B. Eiweisskörper, in Betracht kommen<sup>1)</sup>.

Die Möglichkeit, diese Ansicht zu prüfen, bot sich im Anschluss an Beobachtungen über die Entschwellung des Muskels unter dem Einfluss von Säure und Salzen, die ich im Jahre 1899 zuerst veröffentlicht habe<sup>2)</sup>. Ich hatte gefunden, dass der Froschmuskel durch einen gewissen Säurezusatz in einer isotonischen NaCl-Lösung zum Schwellen gebracht wird; dass er ferner in einer neutralen, aber stark hypertonen Lösung zwar zuerst Wasser verliert, schliesslich aber Wasser aufnimmt und ebenfalls schwillt. Bringt man aber den Muskel in eine Mischung von hypertoner Salzlösung und Säure, so tritt eine Entschwellung ein. Ich erwähnte schon damals die Möglichkeit, dass diese Erscheinung auf die Wirkung der Stoffe auf die Eiweisskörper des Muskels zurückzuführen sei. Diese Vermutung ist nun inzwischen durch die Versuche von Pauli und seinen Schülern, sowie von Prokter zur Gewissheit erhoben worden.

In den Arbeiten dieser Autoren über den Einfluss der Säuren auf die physikalischen Eigenschaften der Eiweisskörper zeigte es sich nämlich, dass das Anion der Säure von grossem Einfluss ist, dass HCl beispielsweise viel wirksamer ist als H<sub>2</sub>SO<sub>4</sub>. Ich veranlasste

<sup>1)</sup> Science 34, 653, 1912.

<sup>2)</sup> Arch. f. d. ges. Physiol. 75, 308, 1899. Vorlesungen über die Dynamik der Lebenserscheinungen S. 77, 1906.

nun Herrn Dr. Beutner, festzustellen, welche Konzentration der Salzlösung erforderlich ist, um die schwellende Wirkung der Säure mit demselben Anion auf den Froschmuskel zu verhindern. Es zeigte sich, dass die entswellende oder antagonistische Wirkung von  $\text{Na}_2\text{SO}_4$  auf  $\text{H}_2\text{SO}_4$  erheblich viel grösser ist als die entswellende Wirkung von  $\text{NaCl}$  auf  $\text{HCl}$ . „Bestimmt man diejenige Konzentration des  $\text{Na}_2\text{SO}_4$ , die in einer beispielsweise  $\frac{n}{200}$ - $\text{H}_2\text{SO}_4$ -Lösung aufzuwenden ist, um nach ca. 24 Stunden weder Schwellung noch Entswellung hervorzubringen, so findet man, dass dieselbe zwischen 0,01 und 0,02 mol.  $\text{Na}_2\text{SO}_4$  liegt.“ Bestimmt man die Konzentration von  $\text{NaCl}$ , die nötig ist, um eine schwellende Wirkung von  $\frac{n}{200}$ - $\text{HCl}$  zu hemmen, so findet man, dass dieselbe zwischen  $\frac{5}{16}$  und  $\frac{6}{16}$  m- $\text{NaCl}$  liegt<sup>1)</sup>. Die antagonistische Wirkung von  $\text{Na}_2\text{SO}_4$  ist also für  $\frac{n}{200}$ -Säure mindestens zomal so gross wie die von  $\text{NaCl}$ . Hier handelt es sich um eine spezifische Eiweissreaktion.

Im folgenden wollen wir nun den Nachweis führen: erstens, dass Säure die Durchgängigkeit der Eihaut von *Fundulus* rasch erhöht; zweitens, dass diese Durchgängigkeitserhöhung durch Säure durch Salze gehemmt wird; drittens, dass diese antagonistische Wirkung für das System  $\text{H}_2\text{SO}_4$  —  $\text{Na}_2\text{SO}_4$  viel grösser ist als für das System  $\text{HCl}$  —  $\text{NaCl}$ .

Die Methode bestand darin, dass die Eier, nachdem sie zweimal in destilliertem Wasser gewaschen waren, in eine Säurelösung resp. Säure-Salzlösung gebracht wurden und nach verschiedenen Zeiten je 4 Eier herausgenommen, durch Waschen in Seewasser von der Säure befreit und dann in die Probeflösung 50 ccm 3 m- $\text{NaCl}$  + 1 ccm  $2\frac{1}{2}$  m- $\text{CaCl}_2$  gebracht wurden. Wie erwähnt, schwimmen in dieser „Probeflösung“ die normalen Eier etwa 3 Tage lang an der Oberfläche. Eine kurze Säurebehandlung erhöht aber die Durchgängigkeit der Eier sehr rasch.

Eier von *Fundulus*, die mehrere Tage vorher befruchtet waren, wurden in eine  $\frac{n}{1250}$ - $\text{H}_2\text{SO}_4$ -Lösung gebracht. Nach verschiedenen Intervallen wurden je 4 Eier in die Probeflösung übertragen und festgestellt, wie lange sie oben schwammen.

<sup>1)</sup> Beutner, diese Zeitschr. 39, 280, 1912.



TABELLE X.

Nach Stunden	Zahl der in der Probeflösung oben schwimmenden Eier.									
	Vorbehandelt mit n/1250-H <sub>2</sub> SO <sub>4</sub>									
	4	8	12	16	20	25	31	37	48	60 Min.
2	4	4	3	1	3	2	2	0	0	0
3½	3	2	0	0	0	0	0			
7½	2	0								
23	0									

Gleichzeitig wurde ein Parallelversuch mit denselben Eiern in einer  $n/1250\text{-H}_2\text{SO}_4 + m/2\text{-Na}_2\text{SO}_4$  angestellt; d. h. die  $n/1250\text{-H}_2\text{SO}_4$ -Lösung war mit einer  $m/2\text{-Na}_2\text{SO}_4$ -Lösung statt wie vorhin mit destilliertem Wasser angestellt. Wie die folgende Tabelle zeigt, tritt nunmehr die Erhöhung der Durchlässigkeit der Eihaut gar nicht oder nur in einem viel geringeren Grade ein.

TABELLE XI.

Nach	Zahl der in der Probeflösung oben schwimmenden Eier.									
	Vorbehandlung in n/1250-H <sub>2</sub> SO <sub>4</sub> in m/2-Na <sub>2</sub> SO <sub>4</sub> von									
	5	10	20	30	40	60	80	100	120	240 Min.
7½ Std.	4	4	4	4	4	4	4	4	4	4
23½ "	3	3	3	2	3	4	2	1	1	1
32 Tagen	1	2	2	1	2	1	2	0	0	1
"	1	2	2	1	1	1	0			1

Während eine  $n/1250\text{-H}_2\text{SO}_4$ -Lösung ohne Na<sub>2</sub>SO<sub>4</sub> die Durchgängigkeit der Eihaut schon nach 20 Minuten so weit erhöhte, dass die Eier in weniger als 3½ Stunden in der Probeflösung zu Boden sanken, hemmte die Gegenwart der  $m/2\text{-Na}_2\text{SO}_4$ -Lösung diese Säurewirkung so weit, dass nach einem Aufenthalt von 20 Minuten in der Säure-Salzlösung diese Eier z. B. noch nach 3 Tagen an der Oberfläche der Probeflösung schwammen.

TABELLE XII.

Nach	Zahl der in der Probeflösung oben schwimmenden Eier.				
	Vorbehandelt mit n/625-H <sub>2</sub> SO <sub>4</sub>				
	7	13	18	25	37 Min.
1 Std.	4	3	1	0	0
2½ Std.	0	0	0		

Es wurde nun eine Versuchsreihe mit der doppelten Konzentration von  $\text{H}_2\text{SO}_4$  angestellt; wir lassen zunächst den Versuch in der reinen  $n/625\text{-H}_2\text{SO}_4$  (ohne Salz) folgen.

Schon nach 7 Minuten erhöht eine  $n/625\text{-H}_2\text{SO}_4$ -Lösung die Durchgängigkeit der Eihaut so erheblich, dass die Eier in der Probeklösung schon in weniger als  $2\frac{1}{2}$  Stunden zu Boden sinken.

In Gegenwart einer  $m/2\text{-Na}_2\text{SO}_4$ -Lösung wird in einer  $n/625\text{-H}_2\text{SO}_4$ -Lösung die Durchgängigkeit der Eihaut in  $6\frac{1}{2}$  Minuten nicht merklich erhöht.

TABELLE XIII.

Nach	Zahl der in der Probeklösung oben schwimmenden Eier.							
	Vorbehandelt mit $n/625\text{-H}_2\text{SO}_4$ in $m/2\text{-Na}_2\text{SO}_4$							150 Min.
	$6\frac{1}{2}$	11	22	33	42	56	80	
$2\frac{1}{2}$ Std.	4	4	4	4	4	4	4	4
25 "	4	4	0	0	0	0	0	0
2 Tagen	2	1						
3 "	2	0						
4 "	0							

Wir wollen nun damit die Wirkung von  $\text{HCl}$  und die Hemmung dieser Wirkung durch  $\text{NaCl}$  vergleichen. Zunächst wurde die Wirkung einer reinen  $n/1250\text{-HCl}$ -Lösung untersucht.

TABELLE XIV.

Nach	Zahl der in der Probeklösung oben schwimmenden Eier.						
	Vorbehandelt mit $n/1250\text{-HCl}$						57 Min.
	5	9	$12\frac{1}{2}$	$16\frac{1}{2}$	21	30	
$2\frac{1}{2}$ Std.	4	4	2	1	0	0	0
$4\frac{1}{2}$ "	3	3	0	0			
23 "	1	0					

Die Wirkung der  $n/1250\text{-HCl}$ -Lösung ist nur wenig kräftiger als die der  $n/1250\text{-H}_2\text{SO}_4$ -Lösung.

Gleichzeitig wurde ein Versuch mit  $n/1250\text{-HCl} - m/2\text{-(NaCl + KCl + CaCl}_2\text{)}$  angestellt.

Wenn man Tabelle XV mit XI vergleicht, in der der entsprechende  $\text{H}_2\text{SO}_4 - \text{Na}_2\text{SO}_4$ -Versuch mitgeteilt wird, so wird man bemerken, wie viel geringer die Hemmung der Wirkung von  $n/1250\text{-}$

TABELLE XV.

Nach	Zahl der in der Probeflösung oben schwimmenden Eier. Vorbehandelt mit $n/1250$ -HCl in $m/2$ -(NaCl + KCl + CaCl <sub>2</sub> )							
	5	10	20	30	40	60	84	100 Min.
4 Stunden	4	4	4	2	0	2	3	1
6 " "	4	4	4	1		2	1	0
23 " "	4	1	0	0		0	0	

HCl durch  $m/2$ -(NaCl + KCl + CaCl<sub>2</sub>) als diejenige von  $n/1250$ -H<sub>2</sub>SO<sub>4</sub> durch  $m/2$ -Na<sub>2</sub>SO<sub>4</sub> ist. Im letzteren Falle war die Hemmung so stark, dass ein Aufenthalt von 20 Minuten in der Säurelösung die Durchgängigkeit der Eier kaum erhöhte, während im System  $n/1250$ -HCl —  $m/2$ -(NaCl + KCl + CaCl<sub>2</sub>) bereits nach 20 Minuten die Durchgängigkeit der Eier erheblich erhöht wurde. Es besteht zwar ein Antagonismus zwischen HCl und NaCl, derselbe ist aber viel geringer als der Antagonismus zwischen H<sub>2</sub>SO<sub>4</sub> und Na<sub>2</sub>SO<sub>4</sub>.

Es wurden schliesslich noch Versuche mit der Wirkung von Essigsäure und Essigsäure in  $m/2$ -(NaCl + KCl + CaCl<sub>2</sub>) angestellt. Tabellen XVI und XVII geben das Resultat.

TABELLE XVI.

Nach	Zahl der in der Probeflösung oben schwimmenden Eier. Vorbehandelt mit 1,5 ccm $n/10$ -Essigsäure + 50 ccm H <sub>2</sub> O							
	4	8	12	17	22	29	37	46 Min.
7 Stunden	4	3	2	2	0	0	0	0
22 " "	3	2	0	0				

TABELLE XVII.

Nach	Zahl der in der Probeflösung oben schwimmenden Eier. Vorbehandelt mit 1,5 ccm $n/10$ -Essigsäure + 50 ccm $m/2$ -(NaCl + KCl + CaCl <sub>2</sub> )						
	9	15	25	35	51	98	192 Min.
7 Stunden	4	4	4	4	4	4	4
22 " "	3	4	4	4	1	4	3
2 Tagen	1	1	1	1	0	1	1

Die hemmende Wirkung der Salzlösung ist sehr schlagend.  
Alle diese Versuche machen es wahrscheinlich, dass die Erhöhung

der Durchlässigkeit der Eihaut unter dem Einflusse von Säuren und deren Hemmung durch die gleichzeitige Gegenwart von Salzen durch eine Wirkung von Säuren und Salzen auf Eiweisskörper zustande kommt. Denn wir kennen heute keine andere Gruppe von chemischen Körpern, die ein derartiges Verhalten zeigt.

Diese Versuche werfen auch ein Licht auf Versuche über den Antagonismus von Säuren und Salzen, die Wasteneys und ich vor kurzem mitgeteilt haben. Wir fanden, dass eine Säurelösung in niedriger Konzentration das Epithel von Fischen stark verändert, wodurch dasselbe in Fetzen abfällt, während der Zusatz von Salzen diese Wirkung verhindert. Wir nahmen an, dass die Säure allein die eiweisshaltige Oberflächenlamelle der Zellen zum Quellen bringt und infolgedessen die Durchgängigkeit der Oberflächenlamelle erhöht. Dadurch kann Säure in die Zellen dringen und dieselben töten. Ist aber ausser der Säure auch Salz zugegen, so kann, infolge der kombinierten Wirkung von Säure und Salz, die Säure nicht mehr in die Zellen diffundieren, wodurch diese dann am Leben bleiben. Unsere neuen Versuche liefern nun den Beweis, dass in der Tat die Säure allein die Durchgängigkeit der Eihaut für Säure erhöht, während die Gegenwart von Salzen diese Erhöhung der Durchgängigkeit hemmt.

Damit stehen nun die folgenden Beobachtungen im Einklang. Bringt man die Eier von *Fundulus* in eine  $\frac{n}{625}$ -H<sub>2</sub>SO<sub>4</sub>-Lösung, so steht bei allen Embryonen in weniger als  $1\frac{1}{2}$  Stunden das Herz still. Bringt man die Eier aber in  $\frac{n}{625}$ -H<sub>2</sub>SO<sub>4</sub> in  $\frac{m}{2}$ -Na<sub>2</sub>SO<sub>4</sub>, so ist nach  $1\frac{1}{2}$  Stunden bei allen Embryonen die Zirkulation im Gange, und selbst nach 15 Stunden findet man noch Embryonen mit intakter Zirkulation.

In  $\frac{n}{1250}$ -H<sub>2</sub>SO<sub>4</sub> steht die Zirkulation in wenigen Stunden still. In einer  $\frac{n}{1250}$ -H<sub>2</sub>SO<sub>4</sub>-Lösung in  $\frac{m}{2}$ -Na<sub>2</sub>SO<sub>4</sub> leben die Embryonen dauernd. Ebenso tötet  $\frac{n}{333}$ -Essigsäure alle die Embryonen ziemlich rasch, während in derselben Konzentration von Essigsäure in einer  $\frac{m}{2}$ -Lösung von NaCl + KCl + CaCl<sub>2</sub> die Embryonen dauernd am Leben bleiben. In der reinen Säurelösung erhöhte die Säure rasch die Durchgängigkeit der Membran, Säure diffundierte in das Ei und erreichte den Embryo; das zeigte sich darin, dass nach 3 bis 5 Minuten in einer  $\frac{n}{1250}$ -HCl- oder H<sub>2</sub>SO<sub>4</sub>-Lösung die Em-

bryonen plötzlich sehr unruhig wurden und lebhafte Bewegungen ausführten. Die Salzlösung hemmte diese Erhöhung der Permeabilität der Membran, und die Embryonen blieben ruhig und am Leben.

Der direkte Nachweis der Erhöhung der Durchgängigkeit der Membran durch Säuren, sowie der Nachweis der Hemmung dieser Erhöhung der Durchgängigkeit durch Salze und endlich der Nachweis, dass diese Hemmung im System  $\text{H}_2\text{SO}_4 - \frac{m}{2}\text{-Na}_2\text{SO}_4$  vollständiger ist als im System  $\text{HCl} - \frac{m}{2}\text{-(NaCl + KCl + CaCl}_2\text{)}$ , zwingt uns zur Annahme, dass die Erhöhung der Durchlässigkeit der Eihaut durch Säure durch eine Beeinflussung der Eiweisskörper der Membran durch die Säure bestimmt ist.

Man kann nun die Frage aufwerfen, ob Anzeichen dafür vorhanden sind, dass auch die Erhöhung der Durchgängigkeit der Membran durch Salze auf eine Modifikation von Eiweisskörpern in der Membran zurückgeführt werden kann. Vielleicht kann die folgende Erfahrung in diesem Sinne verwertet werden. Wenn man sich Lösungen von  $\text{MgCl}_2$ ,  $\text{MgBr}_2$  und  $\text{MgSO}_4$  von  $\frac{8}{8}$  m bis  $\frac{20}{8}$  m herstellt und Eier von *Fundulus* in dieselben bringt, so beobachtet man, dass in den Lösungen von  $\text{MgCl}_2$  und  $\text{MgBr}_2$  die Eier in ganz kurzer Zeit eine starke Erhöhung ihrer Durchgängigkeit erleiden. Der Dottersack schrumpft, die Embryonen sterben, und die Eier sinken zu Boden. In den  $\text{MgSO}_4$ -Lösungen geht dieser Prozess sehr viel langsamer vor sich. In einer  $\frac{18}{8}$  m- $\text{MgSO}_4$ -Lösung waren beispielsweise die Embryonen noch nach  $8\frac{1}{2}$  Stunden am Leben, während sie in  $\frac{10}{8}$  bis  $\frac{20}{8}$  m- $\text{MgBr}_2$  schon in 40 Minuten alle tot waren. In  $\text{MgCl}_2$  verlief der Versuch nicht wesentlich anders wie in  $\text{MgBr}_2$ . Es handelt sich hier um eine spezifische  $\text{SO}_4$ -Wirkung. Der Umstand, dass auch hier  $\text{SO}_4$  die Durchgängigkeit nicht so rasch erhöht wie Cl und Br, steht vielleicht im Zusammenhange mit der Erfahrung, dass im System  $\text{HCl} - \text{NaCl}$  die Erhöhung der Durchgängigkeit durch Säure weniger stark gehemmt ist als im System  $\text{H}_2\text{SO}_4 - \text{Na}_2\text{SO}_4$ .

Ich beabsichtige, diese Versuche weiter auszudehnen, möchte aber hier darauf hinweisen, dass vielleicht unsere Versuche den von Overton gefundenen Unterschied in der Wirkung von  $\text{K}_2\text{SO}_4$  und KCl auf den Muskel begreiflich erscheinen lassen.

# IX. DER EINFLUSS VERSCHIEDENER ALKOHOLE AUF DIE DURCHGÄNGIGKEIT DER EIHAUT VON FUNDULUS.

Für die physiologische Wirkung der Alkohole ist das Gesetz charakteristisch, dass jeder höhere Alkohol einer Reihe etwa dreimal so wirksam ist wie das vorausgehende Glied der Reihe. Dieses Gesetz weist auf eine Beziehung zwischen der Wirksamkeit der Alkohole und ihrer Lipoidlöslichkeit hin. Wir kennen keine derartige Beziehung zwischen Eiweisskörpern und Alkoholen. Es war deshalb von Interesse, zu ermitteln, ob erstens die Alkohole auch die Durchlässigkeit der Eihaut von Fundulus erhöhen, und ob zweitens die relative Wirksamkeit der verschiedenen Alkohole in dieser Hinsicht sich in eine Reihe nach Potenzen von 3 ordnen lässt. Das ist nun in der Tat der Fall, obwohl das Ende der Laichperiode nicht erlaubte, so viele Versuche anzustellen, als wir wünschten.

Die Alkohole wurden in Wasser gelöst, und die Eier wurden in einem verschlossenen Gefäss der Alkoholwirkung ausgesetzt. Nach verschiedenen Intervallen wurden je 4 Eier aus der Alkohollösung genommen, in Reagensgläser mit der Probelösung (50 ccm 3 m-NaCl + 1 ccm 2<sup>1</sup>/<sub>2</sub> m-CaCl<sub>2</sub>) gebracht und ermittelt, wie lange sie an der Oberfläche schwimmen. Der folgende Versuch mit grammolekularem Äthylalkohol soll als Beispiel der Methode und des Versuchsverlaufes dienen.

TABELLE XVIII.

Nach	Zahl der oben schwimmenden Eier in der Probelösung.					
	Vorbehandelt mit m-Äthylalkohol					60 Min.
	2	5 <sup>1</sup> / <sub>2</sub>	10	20	43	
5 Stunden	4	4	4	3	2	1
8 <sup>1</sup> / <sub>2</sub> "	3	3	3	2	1	0
22 "	2	2	0	0	0	

Es wurden nun ausserdem ähnliche Versuche mit folgenden Alkohollösungen angestellt: 2 m-Methylalkohol; 3 m-Äthylalkohol; <sup>m</sup>/<sub>16</sub>-, <sup>m</sup>/<sub>8</sub>-, <sup>m</sup>/<sub>4</sub>-Normal-Butylalkohol und <sup>m</sup>/<sub>16</sub>-Amylalkohol.

Das Resultat war wie folgt.

Die Durchlässigkeit der Eimembranen wurde ungefähr gleich schnell erhöht durch 2 m-Methylalkohol, m-Äthylalkohol, <sup>m</sup>/<sub>8</sub>-Butylalkohol. Ferner wirkte der <sup>m</sup>/<sub>4</sub> normale Butylalkohol nahezu ebenso schnell wie <sup>m</sup>/<sub>16</sub>-Amylalkohol.

Diese Resultate deuten deutlich genug an, dass die Wirkung der verschiedenen Alkohole in diesem Falle mit ihrer Narkosewirkung parallel verläuft. Wenn es sich im letzteren Falle um Lipoidwirkungen handelt, so sollte das auch bei der Erhöhung der Durchlässigkeit der Eihaut der Fall sein. Nathanson und Höber haben bekanntlich den Gedanken ausgesprochen, dass die Membranen der Zellen aus einem Mosaik aus Eiweisskörpern und Lipoiden bestehen. Eine solche Hypothese würde allen Tatsachen gerecht werden.

Osterhout hat gefunden, dass kleine Mengen von Alkohol den galvanischen Widerstand von *Laminaria* vorübergehend etwas erhöhen, und R. Lillie spricht direkt von einem Antagonismus zwischen Elektrolyten und Narkotiken<sup>1)</sup>. Bezieht man diese Wirkung auf eine Erhöhung der Durchgängigkeit der Membran, so wäre es von Interesse, zu prüfen, ob kleine Mengen von Alkohol auch die Erhöhung der Durchlässigkeit der Eihaut hemmen, welche durch Elektrolyte hervorgebracht wird. Die Versuche wurden in der Weise angestellt, dass Eier von *Fundulus* in Lösungen von 3 m-NaCl gebracht wurden, in denen sie nach wenigen Stunden zu Boden sinken. Zu einer Reihe derartiger Lösungen wurden wachsende

TABELLE XIX.

Nach	Zahl der oben schwimmenden Eier in 50 ccm 3 m-NaCl +							
	0	0,1	0,2	0,4	0,8	1,6	3,2	6,4
	ccm 90 o/o igem (?) Alkohol <sup>2)</sup>							
3 Stunden	4	3	3	4	3	4	4	0
4½ "	1	1	1	2	3	2	2	
6 "	0	0	0	2	2	1	0	
7 "				0	1	0		
8 "					0			

Mengen von Äthylalkohol zugesetzt und beobachtet, ob und bis zu welchem Grade der Alkohol die Zeit des Sinkens der Eier hinauschiebt. Die vorstehende Tabelle gibt das Resultat.

Eine Wiederholung des Versuches ergab ein ähnliches Resultat,

<sup>1)</sup> R. Lillie, Amer. Journ. of Physiol. 29, 372, 1912.

<sup>2)</sup> Der Alkohol hatte lange gestanden und die Flasche war öfters geöffnet worden. Es stellte sich später heraus, dass der Alkohol schwächer war als 90%, aber seine Konzentration war nicht ermittelt worden.

eine geringe Verzögerung des Sinkens der Eier in der Lösung mit 0,4 ccm Alkohol. Es ist möglich, dass hier eine geringe Hemmung der Erhöhung der Durchlässigkeit mittels kleiner Quantitäten Alkohol vorliegt. Die Wirkung ist aber bestenfalls sehr gering.

#### X. DIE WIRKUNG VON ZUCKER UND HARNSTOFF AUF DIE PERMEABILITÄT.

In 3 m-Harnstoff sinken die Eier von *Fundulus* sofort, in 4 m fast sofort. Erst in 5 m-Harnstoff schwimmen die Eier an der Oberfläche. Bringt man die Eier in 5 m-Harnstoff, so werden die Embryonen nach 5 Minuten unruhig und der Dotter beginnt zu schrumpfen; bald darauf sinken die Eier.

Der Verlauf des Versuches wird nicht geändert, wenn man zu 50 ccm 5 m-Harnstoff 1,6 ccm  $2\frac{1}{2}$  m- $\text{CaCl}_2$  zusetzt.

Rohrzucker erhöht die Durchlässigkeit der Eihaut nur sehr langsam. Die Eier schwimmen in Lösungen von 0,6 m und darüber. Je 4 Eier wurden in folgende Lösungen von Rohrzucker gebracht: 0,6, 0,8, 1,0, 1,2, 1,4, 1,6, 1,8, 2,0 m. Selbst in den stärksten Konzentrationen, 2,0 und 1,8 m, waren nach 2 Tagen alle Eier am Leben und keine deutliche Schrumpfung des Dottersackes war eingetreten. Erst nach 4 Tagen wurde hier eine mässige Schrumpfung bemerkbar. In der Zuckerlösung bleibt also die relative Undurchlässigkeit der Membran für Wasser lange erhalten. Eier, die  $1\frac{1}{2}$  Tage lang in einer 2 m-Rohrzuckerlösung gewesen waren, wurden in die Probelösung gebracht (50 ccm 3 m- $\text{NaCl}$  + 1 ccm  $2\frac{1}{2}$  m- $\text{CaCl}_2$ ). Es war keine merkliche Erhöhung der Durchlässigkeit der Eihaut nachweisbar.

#### XI. DIE UMKEHRBARKEIT DER DURCHGÄNGIGKEITSÄNDERUNG DER EIHAUT VON *FUNDULUS*.

Osterhout hat gefunden, dass in einer reinen  $\text{NaCl}$ -Lösung der galvanische Widerstand von *Laminaria* erheblich sinkt, dass die Pflanze aber in Seewasser ihren normalen Widerstand wieder gewinnt. Er hat bei derselben Pflanze 8 Tage hintereinander die Erniedrigung des Widerstandes durch  $\text{NaCl}$  und die Wiederherstellung desselben in Seewasser nachgewiesen. Er schliesst daraus mit



Recht, dass die Änderung der Durchgängigkeit der Membran umkehrbar ist und dass vermutlich umkehrbare Änderungen der Durchgängigkeit eine grosse Rolle im Leben der Pflanzen spielen<sup>1)</sup>).

Auch bei der Eihaut ist die Erhöhung der Durchlässigkeit der Membran durch Säure, Salze und Alkohole ein umkehrbarer Prozess. Wir haben gesehen, dass Salze sowohl wie Säuren imstande sind, die Durchgängigkeit der Eihaut von *Fundulus* rasch zu erhöhen, und die nächste Frage war die nach der Umkehrbarkeit dieser Erscheinung. Das Verhalten der Nieren legt den Gedanken nahe, dass Änderungen der Durchgängigkeit eine Eigenschaft der Nierenzellen und vielleicht aller Zellen sind; wenn das wahr sein sollte, so müssten auch erhebliche Durchgängigkeitserhöhungen der Zellen umkehrbar sein. Die folgenden Versuche zeigen nun, in wie hohem Grade das der Fall ist. Diese Versuche haben aber noch eine andere Bedeutung, indem sie uns helfen, zu bestimmteren Anschauungen über den Mechanismus der beobachteten Durchgängigkeitsänderungen zu gelangen.

Eier wurden durch zweimaliges Waschen in destilliertem Wasser von Seewasser befreit und dann 40 Minuten in 0,4 ccm  $\frac{2}{10}$ -H<sub>2</sub>SO<sub>4</sub> + 50 ccm H<sub>2</sub>O gebracht. Dann wurden sie gewaschen und in Seewasser gebracht. In allen Eiern war die Zirkulation noch im Gange, die Durchlässigkeit ihrer Membran war aber stark erhöht (siehe Tabelle X). Von Zeit zu Zeit wurden nun je vier Eier aus dem Seewasser in die Probelösung (50 ccm 3 m-NaCl + 1 ccm  $\frac{2}{2}$  m-CaCl<sub>2</sub>) übertragen, um zu ermitteln, ob ihre Permeabilität wieder normal geworden sei. Die ersten Eier wurden sofort nach dem Waschen aus dem Seewasser in die Probelösung übertragen; sie sanken in weniger als 2 Stunden; die nächste Partie wurde nach einem Aufenthalt von 40 Minuten im Seewasser in die Probelösung gebracht; diese Eier sanken ebenfalls nach weniger als 2 Stunden. Die folgende Tabelle gibt eine Übersicht über die gesamten Resultate.

Die Eier, die noch später als nach  $4\frac{1}{2}$  Stunden aus dem Seewasser genommen wurden, waren nahezu normal. Wir dürfen also sagen, dass in den ersten 40 Minuten im Seewasser die durch Säure bedingte Erhöhung der Durchlässigkeit der Eihaut nicht rückgängig

<sup>1)</sup> Science 36, 350, 1912.

TABELLE XX.

Dauer des Aufenthaltes der Eier in Seewasser nach der Säurebehandlung	Zeit, die die Eier an der Oberfläche der Probelösung schwimmen
0 Minuten	< 2 Stunden
40 "	< 2 "
165 "	> 4 "
4½ Stunden	> 19 "
	3 Eier mehr als 3 Tage!

wird, dass aber nach rund 3 Stunden schon eine mässige Erholung eingetreten ist, und dass nach 4½ Stunden die Erholung nahezu vollständig ist. Um blossе Adsorptionerscheinungen kann es sich hier wohl nicht handeln, da sonst der Einfluss der ersten 40 Minuten viel stärker sein müsste. Ich beabsichtige aber den Verlauf der Erholung im nächsten Sommer etwas genauer zu untersuchen.

Zur Ergänzung diene folgender Versuch mit HCl. Eier blieben 57 Minuten in 0,4  $\frac{n}{10}$ -HCl + 50 ccm H<sub>2</sub>O, und wurden dann in Seewasser übertragen (siehe Tabelle XIV). Um diese Zeit besaßen etwa 75% der Embryonen noch Herzschlag, bei allen stellte sich aber die Zirkulation wieder ein, nachdem sie einige Zeit im Seewasser gewesen waren. Nach 0, 1½, 4½ und 7 Stunden wurden je vier Eier aus dem Seewasser in die Probelösung gebracht. In 1½ Stunden fand keine nennenswerte Erholung statt, und selbst nach 4½ Stunden war nur bei einem unter den vier untersuchten Eiern eine mässige Erholung eingetreten. Eine deutliche Erholung war erst bei den Eiern bemerkbar, die 7 Stunden lang nach der Säurebehandlung im Seewasser gewesen waren. Nach einem Aufenthalt von 24 Stunden im Seewasser waren die Eier praktisch wieder normal geworden, d. h. sie schwammen mehrere Tage lang an der Oberfläche der Probelösung (50 ccm 3 m-NaCl + 1 ccm 2½ m-CaCl<sub>2</sub>).

Es wurde dann versucht, ob die schädigende Wirkung einer 10/8 m-CaCl<sub>2</sub>-Lösung ebenfalls umkehrbar ist. Eine grosse Zahl von Eiern wurde in eine 10/8 m-CaCl<sub>2</sub>-Lösung gebracht und eine Portion derselben nach 1, 2, 3, 4, 5, 6, 7, 8, 10, 13, 15 und 16 Minuten in Seewasser übertragen. Alle blieben 24 Stunden lang in dem Seewasser und wurden dann in die hypertonsche Probelösung übertragen. Es zeigte sich, dass ein Teil der Eier (nicht alle), die 13

Minuten oder weniger der  $10/8$  m-CaCl-Lösung ausgesetzt gewesen waren, sich nahezu völlig erholt hatten. Man muss berücksichtigen, dass eine  $10/8$  m-CaCl<sub>2</sub>-Lösung die Durchgängigkeit der Eihaut rascher schädigt als eine  $n/1250$ -Lösung von HCl oder H<sub>2</sub>SO<sub>4</sub>.

Auch die schädigende Wirkung des Alkohols ist umkehrbar. Eier waren 91 Minuten in eine Lösung von Äthylalkohol gebracht worden, deren Konzentration durch ein Versehen nicht genau bestimmt wurde, die aber vermutlich ein wenig stärker als grammolekular war. Die Eier wurden dann in Seewasser übertragen, und nach 0, 7, 30, 130 und 292 Minuten wurden je vier Eier in die Probelösung übertragen. Die Eier, die 0, 7 und 30 Minuten im Seewasser gewesen waren, sanken in der Probelösung ungefähr gleich schnell. Drei der Eier aber, die 130 Minuten in dem Seewasser gewesen waren, zeigten vollständige Erholung und schwammen mehrere Tage lang an der Oberfläche der Probelösung.

Diese Versuche mögen genügen, um zu zeigen, dass die Erhöhung der Durchgängigkeit der Eimembranen ein umkehrbarer Vorgang ist.

## XII. ÜBER DIE ROLLE DER SALZE BEI DER UMKEHR DER PERMEABILITÄTSÄNDERUNG.

In den eben beschriebenen Versuchen waren die Eier zur Erholung in Seewasser gebracht worden. Wasteneys und ich hatten gefunden, dass *Fundulus* grössere plötzliche Temperaturerhöhungen ertragen können, wenn der Fisch im Seewasser, als wenn er sich in destilliertem Wasser befindet<sup>1)</sup>. Da die Möglichkeit vorlag, dass hierbei Änderungen der Permeabilität des Epithels eine Rolle spielen, so stellte ich Versuche darüber an, ob sich die *Funduluseier* in destilliertem Wasser und in verschiedenen Salzlösungen ebenso rasch erholen als im Seewasser.

Diese Versuche ergaben nun das merkwürdige Resultat, dass in destilliertem Wasser die Eier mit geschädigter Membran zwar dauernd am Leben bleiben, sich weiter entwickeln und ausschlüpfen, dass aber die Änderung der Durchlässigkeit im destillierten Wasser praktisch nicht umkehrbar ist.

<sup>1)</sup> Journ. of Exper. Zoology 12, 543, 1912.

In all den Versuchen über Reversibilität, die im vorigen Abschnitt erwähnt wurden, wurden immer Parallelversuche derart angestellt, dass die eine Hälfte der Eier nach der Behandlung mit Säure oder Alkohol in Seewasser, die andere Hälfte in destilliertes Wasser gebracht wurde, um zu prüfen, ob sich hier die normale Undurchlässigkeit der Eihaut in beiden Fällen gleich schnell wieder herstellen würde.

Eier wurden 57 Minuten lang einer  $\text{N}/_{1250}\text{-HCl}$ -Lösung ausgesetzt und dann wurde ein Teil in Seewasser, der Rest in destilliertes Wasser gebracht, um zu ermitteln, ob in beiden Lösungen die Erholung gleich schnell vonstatten ginge. Nach 22 Stunden wurden je 4 Eier in die Probelösung gebracht. Die Eier, die 22 Stunden im Seewasser gewesen waren, hatten sich völlig erholt, sie schwammen zum Teil 4 Tage lang in der Probelösung. Die Eier aber, die 22 Stunden im destillierten Wasser gewesen waren, sanken schon in weniger als 7 Stunden in der Probelösung. Im destillierten Wasser war also die durch Säure verursachte Erhöhung der Durchlässigkeit der Membran nur zu einem geringen Teil rückgängig geworden. Wenn die Eier 2 bis 3 Tage zur Erholung in destilliertem Wasser blieben, so sanken sie noch rascher in der Probelösung. Man gewinnt also den Eindruck, dass in destilliertem Wasser anfangs eine geringe Erholung stattfindet, die aber bei längerem Aufenthalt wieder schwindet.

Ein Teil der Eier, die 40 Minuten lang mit  $\text{N}/_{1250}\text{-H}_2\text{SO}_4$  behandelt waren, wurde in Seewasser, der Rest in destilliertes Wasser gebracht. Nach 21 Stunden wurden 4 Eier aus dem destillierten Wasser in die Probelösung übertragen. Nach weniger als 5 Stunden waren alle diese Eier am Boden. Die zur Erholung in Seewasser gebrachten Eier schwammen zum Teil noch nach 4 Tagen an der Oberfläche der Probelösung. Auch wenn die Eier zur Erholung mehrere Tage in dem destillierten Wasser blieben, so trat keine Umkehr der in der Säurelösung erfolgten Erhöhung der Durchlässigkeit ein. Die Eier aber waren sonst völlig normal und entwickelten sich in destilliertem Wasser weiter.

Genau so verhielten sich die Eier, deren Durchlässigkeit durch andere Stoffe, nämlich  $\text{HgCl}_2$  oder Alkohol, erhöht war. Wurden die Eier aus diesen Lösungen genommen, ehe die Embryonen tot

waren, als aber die Durchlässigkeit der Membran bereits stark erhöht war, so trat nach einigen Stunden eine Erholung der Eihaut ein, wenn die Eier in Seewasser gebracht wurden; wurden aber die Eier in destilliertes Wasser gebracht, so trat anfangs eine sehr geringe Erholung ein, die aber alsbald wieder völlig zurückging.

Es entstand nun die Frage, ob die Alkalinität des Seewassers oder der Salzgehalt für diese Erholung verantwortlich sei. Versuche ergaben, dass die Eier sich in einer neutralen  $\frac{m}{2}$ -Lösung von  $\text{NaCl} + \text{KCl} + \text{CaCl}_2$  ungefähr ebenso rasch und vollständig erholen wie in Seewasser.

Wir gewinnen den Eindruck, dass im Leben der Zelle Änderungen der Durchlässigkeit häufig eintreten, dass dieselben aber umkehrbar sind und dass diese Umkehrbarkeit von der Gegenwart von Salzen herrührt.

### XIII. DIE ERHÖHUNG DER DURCHLÄSSIGKEIT DER EIMEMBRAN DURCH DESTILLIERTES WASSER.

Die im vorigen Abschnitt erwähnten Versuche deuteten bereits darauf hin, dass das destillierte Wasser selbst die Durchgängigkeit der Eihaut erhöht. Das würde deshalb auffällig sein, weil die Eier von *Fundulus* sich in destilliertem Wasser völlig normal entwickeln und ausschlüpfen können<sup>1)</sup>. Es wurde nun folgender einfache Versuch ausgeführt: Eine Kultur von Eiern wurde 2 Tage nach der Befruchtung in 2 Teile geteilt. Ein Teil der Eier blieb im Seewasser, der Rest wurde in destilliertes Wasser übertragen. Nach verschiedenen Intervallen wurden je 4 Eier in die Probelösung übertragen und festgestellt, wie lange die Eier oben schwimmen. Tabelle XXI gibt das Resultat für destilliertes Wasser.

Die Eier, die im Seewasser geblieben waren, schwammen in der Probelösung wie gewöhnlich 3 Tage oder länger. Nur gegen die Zeit des Ausschlüpfens wird auch hier die Durchgängigkeit der

<sup>1)</sup> Man muss bei Versuchen in destilliertem Wasser die Vorsicht gebrauchen, die abgestorbenen Eier sofort zu entfernen, da sich sonst rasch hier Pilze bilden, die die noch lebenden Eier infizieren. Das Übersehen dieses Umstandes hat wohl einige Autoren zu dem irrigen Schluss geführt, dass die *Funduluseier* nicht in destilliertem Wasser ausschlüpfen.

TABELLE XXI.

Dauer des Aufenthaltes der Eier in dest. Wasser	Zeit, die die Eier an der Oberfläche der Probeflösung schwimmen
1 Tag	>34 <48 Stunden
2 Tage	>10 <24 Stunden
3 Tage	>10 <24 Stunden
4 Tage	2 sinken in weniger als 10 Stunden, der Rest " " " 24 "
5 Tage	3 sinken in weniger als 8 Stunden, das vierte " " " 24 "
7 Tage	6 Stunden
8 Tage	4 Stunden

Membran erhöht. Es ist also sicher, dass die Durchlässigkeit der Eihaut durch das destillierte Wasser langsam, aber stetig zunimmt<sup>1)</sup>. Dass Mikroorganismen hierfür verantwortlich sein könnten, ist möglich, aber nicht sehr wahrscheinlich, weil die Resultate so regelmässig waren. Es ist auch möglich, dass es sich hier nicht oder nicht bloss um eine Wirkung des destillierten Wassers selbst, sondern um eine Wirkung der CO<sub>2</sub> handelt; im Seewasser wird die Säure neutralisiert, und selbst in einer neutralen Salzlösung hemmt die Gegenwart des Salzes die Säurewirkung auf die Membran.

#### XIV. DAS VERHALTEN TOTER EIER.

Es ist lange bekannt, dass die Durchlässigkeit der Zellen mit dem Tode erhöht wird. So lange es sich um die Durchlässigkeit des Protoplasmas handelt, ist das leicht zu verstehen. Es interessierte mich, wie es sich mit der Eihaut des Fischembryos verhält, die ja nur eine lose Hülle um den Embryo und Dotter bildet und mit dem letzteren in keinem direkten Zusammenhang steht. Es wurden Versuche mit einer 4<sup>0</sup>/<sub>10</sub>igen Formaldehydlösung gemacht, die als Konservierungsflüssigkeit für Tiere dient. Nach 25 Minuten standen die Herzen der Embryonen still, zum Zeichen, dass ein wenig der Lösung schon in das Ei eingedrungen war. Eier, die

<sup>1)</sup> In einem Versuche, in dem eine 20<sup>0</sup>/<sub>10</sub> m-Lösung von (NaCl + KCl + CaCl<sub>2</sub>) als Probeflösung benutzt wurde, war die Erhöhung der Permeabilität der Eihaut nicht so beträchtlich wie in dem vorher zitierten Versuche.

30 Minuten in dieser Lösung gewesen waren, sanken in der Probelösung schon nach 70 Minuten zu Boden. Ein Teil der Eier blieb 24 Stunden in der Lösung und es wurde untersucht, ob nun die Eihaut undurchgängiger geworden sei. Das war aber nicht der Fall; die Eier sanken in der Probelösung in weniger als 50 Minuten zu Boden. In einem anderen Falle wurden Eier 4 Stunden lang in eine  $\frac{1}{1250}$ -HCl-Lösung gebracht. Die Eier waren undurchsichtig und geschwollen, die Säure war also in das Innere gedrungen. Wenn diese Eier in die Probelösung gebracht wurden, so sanken sie in 5 Minuten oder noch schneller. Dabei fand eine kolossale Schrumpfung statt. Die Membran war jetzt für Wasser leicht durchgängig, für Salze aber wohl etwas weniger leicht, daher die Schrumpfung.

In allen diesen Fällen aber ging die Änderung der Durchlässigkeit der Membran dem Tode der Tiere voraus, und so werfen diese Versuche kein Licht auf die Frage, ob das Absterben des Embryos die Durchgängigkeit der Eihaut erhöht, etwa in der Weise, dass der lebende Embryo fortwährend Stoffe produziert, welche zur Erhaltung der Eihaut beitragen, oder dass der Tod des Embryos zur Bildung oder Nichtzerstörung von Stoffen führt, die die Durchgängigkeit der Eihaut erhöhen.

Um diese Frage zu prüfen, wurden eine Reihe von Eiern, die 10 Tage vorher befruchtet waren, in folgende zwei Lösungen, in  $\frac{5}{8}$  m-KCl und in  $\frac{5}{8}$  m-NaCl, verteilt. Während die frisch befruchteten Eier rasch in einer reinen  $\frac{5}{8}$  m-NaCl-Lösung sterben (weil die Membran unmittelbar nach der Befruchtung sehr durchgängig ist), ist dieselbe Lösung einige Tage nach der Befruchtung fast unschädlich, weil inzwischen die Mikropyle oder die Membran im Seewasser hinreichend undurchgängig und widerstandsfähig geworden ist. Die Eier, die in die  $\frac{5}{8}$  m-NaCl-Lösung gebracht wurden, waren in dieser Lösung noch nach 10 Tagen am Leben, d. h. Herzschlag und Zirkulation war bei ihnen erhalten. Bei den Eiern dagegen, die in die  $\frac{5}{8}$  m-KCl-Lösung übertragen wurden, stand die Zirkulation bei allen schon in weniger als 5 Stunden still. Etwas KCl war also durch diese Membran gedrungen und hatte das Herz zum Stillstand gebracht. Nun sterben die Embryonen, wie ich vor 20 Jahren zeigte, nicht sofort, wenn der Herzschlag erlischt, was man dadurch

zeigen kann, dass dieselben sich wieder erholen, wenn sie in normales Seewasser gebracht werden. Aber diese Periode latenten Lebens ist doch beschränkt. Nach verschiedenen Intervallen wurden je 4 Eier aus beiden Lösungen genommen und in die Probelösung (50 ccm 3 m-NaCl + 1 ccm  $2\frac{1}{2}$  m-CaCl<sub>2</sub>) übertragen.

Es zeigte sich nun, dass die Eier, die bis zu 4 Tagen in der  $\frac{5}{8}$  m-NaCl-Lösung gewesen waren, keine Erhöhung der Durchgängigkeit zeigten, wenn sie in die Probelösung gebracht wurden. Sie schwammen 3 Tage oder länger an der Oberfläche. Nach 4 Tagen aber befanden sich die Eier, die in der KCl-Lösung geblieben waren, im Zustand der Fäulnis und die Eier sanken in der Probelösung in 5 bis 10 Minuten. Freilich könnte man das auf eine Erhöhung der Durchgängigkeit der Membran durch Bakterien beziehen. Aber bereits nach einem Aufenthalt von 3 Tagen in der  $\frac{5}{8}$  m-KCl-Lösung zeigten die Eier eine Erhöhung der Durchgängigkeit, indem sie in weniger als 24 Stunden in der Probelösung zu Boden sanken.

Diese Versuche stützen doch vielleicht den Gedanken, dass die Änderung gewisser chemischer Prozesse im Ei, die mit dem Eintritt des Todes stattfindet, auch eine Erhöhung der Durchlässigkeit der Membran herbeiführt.

#### XV. ZUSAMMENFASSUNG DER ERGEBNISSE.

1. Aufgabe der Arbeit war die weitere Prüfung der Theorie antagonistischer Salzlösungen, wonach die antagonistischen Salze die Erhöhung der Durchgängigkeit der Membran hindern, die jedes oder eines der Salze bedingt, wenn es in derselben Konzentration allein in Lösung ist. Diese Prüfung wurde am Ei von *Fundulus* durchgeführt, an dem die Daten für die Aufstellung dieser Theorie seinerzeit gewonnen waren. Als physiologisch äquilibrierte Salzlösungen werden solche Lösungen bezeichnet, in denen diese antagonistische Wirkung ein Maximum ist.

2. Es wird eine Methode zur Untersuchung der Erhöhung der Durchgängigkeit der Eihaut angegeben, die darauf beruht, dass die Eihaut für Wasser und Salze normalerweise undurchgängig ist. Bringt man befruchtete Eier von *Fundulus* in eine Lösung von 50



ccm 3 m-NaCl + 2 ccm  $10/8$  m-CaCl<sub>2</sub>, so schwimmen die Eier etwa 3 Tage an der Oberfläche dieser Lösung. Erst allmählich erhöht diese stark hypertonische Lösung die Durchgängigkeit der Membran und infolgedessen beginnen die Eier nach etwa 3 Tagen zu schrumpfen und infolge der Erhöhung ihres spezifischen Gewichtes zu Boden zu sinken.

3. Bringt man die Eier in eine Lösung nur eines der folgenden Salze, MgCl<sub>2</sub>, CaCl<sub>2</sub>, SrCl<sub>2</sub> und BaCl<sub>2</sub> in Konzentrationen von m bis  $2^1/2$  m, so schrumpfen und sinken die Eier von Fundulus in etwa  $1/2$  Stunde, und zwar um so rascher, je höher innerhalb der angegebenen Grenzen die Konzentration der Lösung ist. Bringt man die Eier in 3 m-Lösungen von NaCl (oder KCl oder LiCl), so sinken dieselben in etwa 3 bis 4 Stunden. Alle diese Lösungen sind nicht äquilibrierte Lösungen. Bringt man die Eier aber in eine Mischung von 50 ccm 3 m-NaCl + 2 ccm  $2^1/2$  m-CaCl<sub>2</sub>, so schwimmen die Eier, wie erwähnt, 3 Tage oder länger in der Lösung.

4. Es lässt sich allgemein zeigen, dass in physiologisch äquilibrierten Salzlösungen die Durchlässigkeit der Eimembran nur sehr langsam, in nicht äquilibrierten Lösungen dagegen sehr rasch erhöht wird. In meinen früheren Versuchen habe ich gezeigt, dass in einer reinen NaCl-Lösung der Embryo von Fundulus rasch getötet wird, dass aber der Zusatz von einer kleinen Menge eines Erdalkalisalzes diese giftige Wirkung des NaCl (oder irgendeines anderen Salzes der Alkalimetalle) aufhebt. Die neuen Versuche zeigen, dass der Zusatz einer bestimmten, aber kleinen Menge eines Salzes der Erdalkalien zu einer 3 m-NaCl-Lösung die Erhöhung der Durchgängigkeit erheblich verzögert. In einer  $2^1/2$  m-Lösung von NaCl + KCl + CaCl<sub>2</sub> schwammen manche Eier 11 Tage lang an der Oberfläche der Lösung, während in einer  $2^1/2$  m-NaCl-Lösung die Eier schon nach etwa 6 Stunden oder noch früher zu Boden sanken.

5. Es wird ein Versuch gemacht zu entscheiden, ob die Eiweisskörper der Eihaut für die Durchgängigkeit der Membran für Wasser und Salze in Betracht kommen. Für Eiweisskörper ist der Antagonismus zwischen Säuren und Salzen charakteristisch und ausserdem die Erfahrung, dass dieser Antagonismus im System H<sub>2</sub>SO<sub>4</sub> — Na<sub>2</sub>SO<sub>4</sub> viel vollständiger ist als im System HCl — NaCl.

Es wird nun in dieser Arbeit gezeigt, dass eine kurze Behandlung der Eier mit Säure die Durchgängigkeit der Eihaut für Wasser (und vielleicht auch für Salze) rasch erhöht; dass Salze diese Erhöhung der Durchgängigkeit der Eihaut hemmen, resp. verzögern, und dass diese Hemmung im System  $\text{H}_2\text{SO}_4 - \text{Na}_2\text{SO}_4$  viel vollständiger ist als im System  $\text{HCl} - \text{NaCl}$ . Daraus wird gefolgert, dass die Erhöhung der Durchgängigkeit der Eihaut für Wasser und Salze durch eine Modifikation der Eiweisskörper der Membran bedingt wird.

6. Wenn dieser Schluss richtig ist, so ist Grund für die Vermutung vorhanden, dass der Austausch von Wasser und wasserlöslichen Bestandteilen zwischen Zellen und umgebender Flüssigkeit eine Funktion der Eiweissbestandteile der Membran ist.

7. Alkohole erhöhen ebenfalls die Durchgängigkeit der Eihaut von Fundulus, und zwar ist die Geschwindigkeit, mit der die verschiedenen Alkohole die Durchlässigkeit erhöhen, für jeden folgenden Alkohol angenähert dreimal so gross wie für den vorausgehenden derselben Reihe. Das weist darauf hin, dass für die Wirkung der Alkohole auf die Durchgängigkeit fettartige Bestandteile der Membran in Betracht kommen.

8. Die Erhöhung der Durchlässigkeit der Membran ist umkehrbar, so lange dieselbe nicht zu weit fortgeschritten ist. Aber eine völlige Erholung des Eies tritt nur in Salzlösungen, z. B. Seewasser resp.  $\frac{m}{2} - (\text{NaCl} + \text{KCl} + \text{CaCl}_2)$  ein. Bringt man die Eier beispielsweise nach einer kurzen Säurebehandlung, die die Durchgängigkeit der Eier erheblich erhöht, aber die Embryonen nicht tötet, in Seewasser, so erholen sich die Eier in wenigen Stunden, d. h. sie sind imstande, an der Oberfläche einer Mischung von 50 ccm 3 m-NaCl + 2 ccm  $\frac{10}{8}$  m- $\text{CaCl}_2$  3 Tage lang zu schwimmen. Bringt man die Eier aber in destilliertes Wasser, so bleiben sie hier am Leben und die Fische können auch ausschlüpfen, aber sie gewinnen ihre Undurchgängigkeit nicht wieder. Wenn man solche Eier aber in 50 ccm 3 m-NaCl + 2 ccm  $\frac{10}{8}$  m- $\text{CaCl}_2$  bringt, so sinken sie in wenigen Stunden.

## DIE OXYDATIONSVORGÄNGE IM BEFRUCHTETEN UND UNBEFRUCHTETEN SEESTERNEI.\*

VON JACQUES LOEB UND HARDOLPH WASTENEYS.

*(Aus den Laboratorien des Rockefeller Institute for Medical Research,  
New York.)*

In früheren Arbeiten über die lebensrettende Wirkung der Befruchtung auf das Ei wies LOEB auf den Zusammenhang des Absterbens des unbefruchteten Eies mit den Oxydationen hin<sup>1)</sup>. Das reife, aber unbefruchtete Seeigelei kann 1 Woche am Leben bleiben, während das reife, unbefruchtete Seesternei in wenigen Stunden zugrunde geht. Entzieht man ihm aber den Sauerstoff oder verzögert man die Geschwindigkeit der Oxydationen im Seesternei durch Zusatz von etwas KCN, so bleibt es erheblich länger am Leben.

Es entstand nun die Frage, warum die reifen, unbefruchteten Seesterneier soviel rascher sterben als die reifen, unbefruchteten Seeigeleier. LOEB sprach die Vermutung aus, dass das vielleicht mit einem Unterschied in der Geschwindigkeit der Oxydationen zusammenhängen könnte. Im befruchteten Seeigelei ist die Geschwindigkeit der Oxydationsprozesse vier- bis sechsmal so gross wie im unbefruchteten Ei, und es wäre denkbar, dass die geringe Oxydationsgeschwindigkeit dem unbefruchteten Ei erlaubt, längere Zeit am Leben zu bleiben; während bei den Eiern anderer Tiere, z. B. des Seesterns, schon im reifen, aber unbefruchteten Ei die Oxydationsgeschwindigkeit eine ebenso hohe oder nahezu so hohe ist wie im befruchteten Ei. »Es ist möglich, dass bei manchen Eiern die Reifung allein schon zu einer erheblichen Beschleunigung der Oxydationen führt und dass diese Eier rasch sterben, wenn sie nicht sofort nach der Reifung befruchtet werden; während bei den Eiern anderer Tiere diese Steigerung der Oxydationsvorgänge unmittelbar nach

\* Eingegangen am 10. September 1912.

<sup>1)</sup> LOEB, PFLÜGERS Archiv. Bd. 93. 1902 u. Archiv f. Entw.-Mech. Bd. 31. 1911. S. 658.

der Reifung geringer ist und dass daher die Eier dieser Tiere nach der Reifung länger am Leben bleiben<sup>1)</sup>). Um die Berechtigung dieser Ansicht zu prüfen, führten wir Messungen des Sauerstoffverbrauches an den befruchteten und unbefruchteten Eiern des Seesterns *Asterias Forbesii* in Woods Hole aus.

#### METHODE DER VERSUCHE.

Die Messung des Sauerstoffverbrauches erfolgte nach der WINKLERSchen Methode. Eine genaue Beschreibung der Anwendung dieser Methode auf diese Probleme findet der Leser in den Arbeiten von O. WARBURG und von uns. Für diese Versuche sind einige besondere Bemerkungen hinzuzufügen. Wir suchten Weibchen aus, deren Eier rasch reiften, stellten eine gleichmässige Suspension der Eier in Seewasser her und teilten die Suspension in zwei gleiche Teile. Zu dem einen wurde Samen zugesetzt, der andre blieb unbefruchtet. Der überschüssige Samen wurde dann sofort durch Waschen entfernt. Beide Portionen von Eiern wurden gleichzeitig in einen Thermostaten gebracht. Während 30 Minuten wurden die Eier in Suspension erhalten (eine Rotation alle 2 Minuten) und dann blieben sie 1 Stunde stehen, um den Eiern zu erlauben sich am Boden abzusetzen. Dieser Prozess geht viel langsamer beim Seestern von statten als beim Seeigellei. Es ist wünschenswert, dass die Masse der Eier nicht zu gross ist. Da es nur ausnahmsweise gelingt, alle Eier eines Seesternweibchens zu befruchten, so wurde nach dem Versuch der Prozentsatz der befruchteten, d. h. sich furchenden Eier durch Zählungen festgestellt. Die Oxydationen wurden erst gemessen, nachdem wir uns überzeugt hatten, dass in der befruchteten Hälfte der Eier die Entwicklung im Gange war.

#### RESULTATE.

Die Resultate geben wir in der folgenden Tabelle:

Versuch	Sauerstoffverbrauch der unbefruchteten Eier mg	Sauerstoffverbrauch der befruchteten Eier mg	Prozentsatz der befruchteten Eier %
I (9./VI.)	0,67	0,51	11
II (11./VI.)	0,64	0,52	20
III (13./VI.)	0,78	0,85	25
IV (16./VI.)	0,34	0,31	71
V (17./VI.)	0,26	0,33	52

<sup>1)</sup> LOEB, loc. cit. S. 661.

Wir dürfen aus diesen Versuchen den Schluss ziehen, dass die Oxydationsvorgänge im reifen, unbefruchteten Ei des Seesterns mit nahezu derselben Geschwindigkeit verlaufen wie im befruchteten Ei.

#### THEORETISCHE BEMERKUNG.

Dieses Resultat entspricht der von LOEB ausgesprochenen Vermutung, dass die Oxydationsvorgänge im Ei, oder gewisse Prozesse, welche von Oxydationsprozessen abhängen, für den raschen Tod des unbefruchteten Eies verantwortlich sind. Das unbefruchtete Ei des Seeigels bleibt danach deshalb länger am Leben, weil die Oxydationen relativ langsam verlaufen. Das unbefruchtete Seesternei geht deshalb rasch zugrunde, weil die Oxydationen in demselben relativ rasch verlaufen. Die lebensrettende Wirkung der Befruchtung besteht in einer Immunisierung des Eies gegen die schädliche Wirkung der Oxydationsvorgänge oder gewisser Vorgänge, welche von Oxydationsvorgängen abhängen. Diese Ansicht wird gestützt durch zwei schon früher erwähnte Tatsachen, nämlich erstens, dass Unterdrückung der Oxydationen das Leben des unbefruchteten Seesterneies verlängert, und zweitens, dass, wenn wir im unbefruchteten Seeigelei durch die künstliche Membranbildung oder durch Zusatz von Alkali die Oxydationen beschleunigen, dasselbe auch rascher abstirbt<sup>1)</sup>.

Diese Untersuchungen unterstützen ferner die oben zitierte Vermutung von LOEB, dass möglicherweise bei den Vorgängen der Reifung und der Ausstossung der Polkörperchen Oxydationsvorgänge von derselben Grössenordnung im Ei stattfinden wie bei der Entwicklung. Es wird sich vielleicht allgemein nachweisen lassen, dass, wenn ein Spermatozoon in ein ruhendes Ei eintritt (wie beim Seeigelei), eine plötzliche Oxydationsbeschleunigung stattfindet; dass aber der Eintritt des Spermatozoons in ein Ei, bei dem eben die Reifeteilungen stattfinden, wie beim Seesternei, keine oder nur eine geringere Oxydationsbeschleunigung herbeiführt.

<sup>1)</sup> Es sei kurz daran erinnert, dass nach LOEB der Vorgang der Entwicklungserregung sich im Seeigelei aus zwei Phasen zusammensetzt; die eine ist die Oberflächenänderung, welche eine Beschleunigung der Oxydationen zur Folge hat. Die zweite besteht in einer Immunisierung des Eies gegen die schädlichen Wirkungen der Oxydationen oder ihrer Folgen.

## EINIGE WEITERE VERSUCHE BETREFFEND OSMOTISCHE UND KOLLOIDALE QUEL- LUNG DES MUSKELS.\*

VON R. BEUTNER.

(Aus den Laboratorien des Rockefeller Institute for Medical Research,  
New York.)

Mit 4 Figuren im Text.

Im folgenden teile ich einige weitere Versuche mit, die auf Veranlassung von Herrn Dr. Jacques Loeb ausgeführt wurden und im Anschluss an eine frühere Arbeit<sup>1)</sup> weitere Erläuterungen, betreffend das osmotische Verhalten des Muskels, geben.

1. Meine Versuche hatten die schon oft vorher erwiesene Tatsache bestätigt, dass osmotische Vorgänge den Wasseraustausch des lebenden Gewebes mit einer umgebenden Lösung regulieren, und zwar unter speziellen Bedingungen (Säure-Salz-Mischungen).

Diejenigen gelösten Stoffe, die in den Gewebsflüssigkeiten osmotischen Druck hervorrufen, sind die Salze, besonders NaCl. Immerhin könnte man noch die Frage aufwerfen, ob nicht auch andere Stoffe, speziell die reichlich gelösten Proteine, einen nennenswerten Bruchteil des osmotischen Druckes ausmachen. Zwar spricht hiergegen schon die allbekannte Tatsache, dass Salzlösung Blut ersetzen kann, ohne Schwellung und Schädigung des Gewebes hervorzurufen; indes ist kürzlich die erstaunliche Behauptung aufgetaucht, dass nicht die Salze, sondern in erster Linie die gelösten Proteine des Blutes den Wasseraustausch mit dem Gewebe regulieren, und zwar indem sie das Wasser „kolloidal binden“<sup>2)</sup>. (?)

\* Eingegangen am 4. December 1912.

<sup>1)</sup> Diese Zeitschr. 39, 280.

<sup>2)</sup> Martin H. Fischer, Kolloidchem. Beihefte 3, 385. Zwar findet auch Fischer, dass reine (gelatinefreie) Salzlösung bei Transfusion unschädlich ist (allerdings durch die Nieren sezerniert wird), dass aber Transfusion salzfreier Gelatinelösung wie destilliertes Wasser tödlich wirkt. Die letztere Wirkung scheint Herrn Fischer schwer verständlich, er sagt hierüber: „Obwohl das Wasser einer solchen (salzfreien) Gelatinelösung an das Kolloid gebunden ist, behält es doch viele Eigenschaften des freien Wassers.“ (1)

Nach Untersuchungen von F. R. Lillie<sup>1)</sup> u. a. besitzen gelöste Proteine einen messbaren osmotischen Druck. Lillie findet als höchsten Wert 30 bis 40 mm für eine etwa 2<sup>0</sup>/<sub>10</sub>ige Albuminlösung, Starling<sup>2)</sup> für die Proteine des Serums 40 mm Hg. Der osmotische Druck einer <sup>m</sup>/<sub>4</sub>-Zuckerlösung oder <sup>m</sup>/<sub>8</sub>-NaCl-Lösung, in der ein Frosch-Gastrocnemius sein Gewicht ungefähr beibehält, ist indes unvergleichlich viel höher, nämlich mehr als 4000 mm Hg, so dass die Proteine höchstens 1<sup>0</sup>/<sub>10</sub> ausmachen können. Der Einfluss auf die Wasseraufnahme des Muskels muss also äusserst klein sein. Der folgende Versuch bestätigt dies.

GEWICHTSVERÄNDERUNG ZWEIER GASTROCNEMII DESSELBEN  
FROSCHES.

	Muskel a in <sup>m</sup> / <sub>8</sub> -Ringerlösung %	Muskel b in <sup>m</sup> / <sub>8</sub> -Ringerlösung + 2% Gelatine %
Nach 1 Std.	-1,6	-2,5
" 3 "	-3,5	-4,3
" 8 "	-3,2	-4,7
" 22 "	-4,4	-3,8
" 29 "	-4,2	-3,8

Die Abweichungen der beiden Zahlenreihen sind im Vergleich mit den Versuchsfehlern gering und zeigen, dass die Gelatine die Wasseraufnahme so gut wie gar nicht beeinflusst.

Zur weiteren Kontrolle wurde konstatiert, dass Muskeln in dialysiertem Blutserum ebenso schwellen wie in einer damit isosmotischen Salzlösung. Verwendet wurde ein 40 Stunden lang in Pergamentpapier dialysiertes Rinderblutserum, das eine Gefrierpunktserniedrigung von 0,03<sup>0</sup> zeigte, entsprechend einem NaCl-Gehalt von ca. <sup>m</sup>/<sub>120</sub>.

Bei den erheblichen Fehlerquellen, die speziell diesen Messungen anhaften, können die beiden Zahlenreihen als identisch angesehen werden. Es ist hiernach klar, dass die Salze und nicht die gelösten Proteine für den Wasseraustausch.massgebend sind.

<sup>1)</sup> F. R. Lillie, Amer. Journ. of Physiol. 20, 197. Siehe auch Moore und Parker, Amer. Journ. of Physiol. 7, 261.

<sup>2)</sup> Starling, Journ. of Physiol. 19, 332; 24, 318.

## GEWICHTSVERÄNDERUNG ZWEIER GASTROCNEMII.

	Muskel a in dialys. Serum %	Muskel b in <sup>m</sup> / <sub>120</sub> -Ringerlösung %
Nach $\frac{1}{2}$ Std.	+22,3	+24,7
" 1 "	+34,0	+38,0
" $2\frac{1}{2}$ "	+51,0	+53,6

Zusatz von Säure erhöht zwar nach Lillies Experimenten<sup>1)</sup> den osmotischen Druck des Proteins, indes selbst bei dialysiertem Serum ist diese Erhöhung zu klein, um die Geschwindigkeit der Wasseraufnahme zu beeinflussen, wie folgende Zahlen zeigen.

## GEWICHTSVERÄNDERUNG ZWEIER MUSKEL.

	Muskel a in dialys. Serum mit Zusatz von 1 ccm <sup>n</sup> / <sub>10</sub> -HCl auf 50 ccm %	Muskel b in dialys. Serum ohne Zusatz von Säure %
Nach 1 Std.	+33,0	+35,0
" 2 "	+43,0	+46,0
" 4 "	+52,0	+49,0
" 6 "	+45,0	+43,0

2. Schon Overton<sup>2)</sup> gibt an, dass die osmotischen Eigenschaften eines Muskels noch längere Zeit nach dem Aufhören der Erregbarkeit bestehen bleiben. Aus meinen Messungen<sup>3)</sup> der Quellbarkeit in Säure-Salzmischungen ergibt sich in einer allerdings nicht besonders klar hervortretenden Art das gleiche. Sehr deutlich zeigen die folgenden Versuche das Vorhandensein osmotischer Wirkung noch sehr lange nach dem Aufhören der Erregbarkeit.

Zwei Gastrocnemii desselben Frosches wurden gewogen und jeder in eine Lösung von 0,05 m-Na<sub>2</sub>SO<sub>4</sub> + 0,001 n-H<sub>2</sub>SO<sub>4</sub> gebracht. Gewichtszunahme nach 10 Stunden:

Muskel a  
+ 32,60/0

Muskel b  
+ 31,60/0

<sup>1)</sup> l. c.

<sup>2)</sup> In seiner ausführlichen Experimentaluntersuchung. Arch. f. d. ges. Physiol. 92, 115.

<sup>3)</sup> l. c.





tion ceteris paribus geringer ist, d. h. die osmotischen Eigenschaften, wie zu erwarten, weitgehender aufgehoben worden sind.

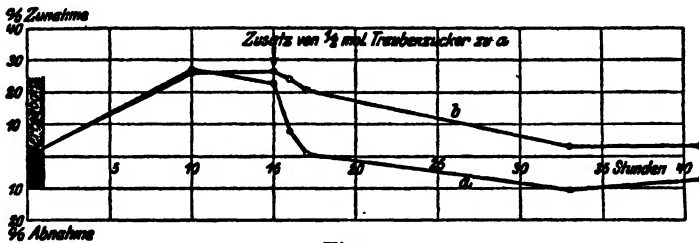


Fig. 2.

Muskeln, die durch Koagulation getötet wurden, zeigen dagegen keinerlei osmotische Eigenschaften, wie folgende Versuche lehren.

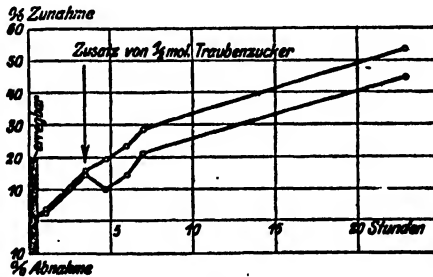


Fig. 3.

Zwei Gastrocnemii in  $\frac{1}{8}$ -NaCl +  $\frac{1}{500}$ -HCl.

Zwei Gastrocnemii desselben Frosches, die in  $\frac{1}{8}$ -Ringerlösung ihr Gewicht nicht änderten, wurden 10 Minuten lang bei  $47^{\circ}$  in  $\frac{1}{8}$ -Ringerlösung digeriert, wodurch sie trüb und steif wurden.

	Muskel a	Muskel b
Gewicht vor der Koagulation:	0,306 g	0,324 g
" nach "	0,283 g	0,304 g

Fig. 4. Zwei Gastrocnemii in  $\frac{1}{8}$ -NaCl +  $\frac{1}{1000}$ -HCl.

# 458 *Weiteres über osmotische und kolloidale Quellung des Muskels.*

Darauf: Muskel a in  $\frac{1}{8}$ -Ringerlösung, Muskel b in  $\frac{1}{16}$ -Ringerlösung bei gewöhnlicher Temperatur.

Nach 5 stündigem Liegen in dieser Lösung wog:

Muskel a	Muskel b
0,253 g	0,257 g
d. h. 10,6% Abnahme.	d. h. 15,1% Abnahme.

In der verdünnten Lösung findet also keine Wasseraufnahme statt (Muskel b), sondern sogar eine noch grössere Wasserabgabe wie in konzentrierterer Lösung; die osmotischen Funktionen sind also zerstört. (Ein Kontrollversuch zeigte, dass ein frischer Muskel in  $\frac{1}{16}$ -Ringerlösung in  $1\frac{1}{4}$  Stunde 22% Wasser aufnahm.)

Auch eine osmotische Schrumpfung in hypertonischer Lösung war bei einem koagulierten Muskel experimentell nicht zu konstatieren. Hiernach scheint das Unerregbarmachen eines Muskels durch Wärme und durch typische Gifte (Säuren) auf wesentlich verschiedenen Vorgängen zu beruhen, da im ersten Falle die osmotischen Funktionen sofort verschwinden, im letzten Falle dagegen noch lange erhalten bleiben.

Es war nun noch von Interesse, zu prüfen, inwieweit der koagulierte Muskel die typisch kolloidalen Quellungerscheinungen zeigt (d. h. diejenigen, die man auch an Gelatineplättchen beobachtet).

## WASSERAUFNAHME ZWEIER BEI 47° KOAGULIERTEN GASTROCNEMI DESSELBEN FROSCHES.

	Muskel a in $\frac{1}{8}$ -NaCl + $\frac{1}{1000}$ -HCl %	Muskel b in $\frac{1}{8}$ -NaCl + $\frac{1}{1000}$ -HCl %
Nach 1 Std.	- 2,1	+ 0,5
" 2 $\frac{1}{2}$ "	-10,0	+ 2,2
" 6 "	-10,0	+ 2,0
" 23 "	- 9,5	+11,8
" 30 "	- 6,5	+19,5

Diese Zahlen sind innerhalb der Fehler, die durch Verschiedenheit des physiologischen Materials bedingt sind, identisch mit meinen

früheren Versuchen<sup>1)</sup>), betreffend Wasseraufnahme nicht koagulierter Muskeln in den gleichen Lösungen. Dies ist zu erwarten, da die osmotischen Eigenschaften des Muskels speziell bei NaCl—HCl-Mischungen nicht zutage treten können, wie ich in meiner früheren Arbeit begründet habe. Die ent quellende Wirkung der höheren NaCl-Konzentration ist somit bei beiden Versuchsreihen durch kolloidale Vorgänge bedingt.

Dagegen lassen sich in Na<sub>2</sub>SO<sub>4</sub>—H<sub>2</sub>SO<sub>4</sub>-Mischungen, wie früher ausgeführt, die osmotischen Quellungserscheinungen getrennt erkennen, z. B. in 0,05 m-Na<sub>2</sub>SO<sub>4</sub> + 0,001 n-H<sub>2</sub>SO<sub>4</sub> an einer osmotischen Wasseraufnahme, auf die eine kolloidale Wasseraufnahme zeitlich folgt. Beim koagulierten Muskel müsste sofort eine Wasserabgabe durch kolloidale Vorgänge beobachtet werden. Dies ist auch der Fall, wie die folgenden Zahlen zeigen.

GEWICHTSVERÄNDERUNG EINES KOAGULIERTEN MUSKELS.

			In $\frac{1}{20}$ -Na <sub>2</sub> SO <sub>4</sub> + $\frac{1}{1000}$ -H <sub>2</sub> SO <sub>4</sub> %
Nach	1	Std.	— 3,4
"	$2\frac{1}{2}$	"	— 3,4
"	6	"	— 14,9
"	23	"	— 17,0
"	30	"	— 17,4

P. S. Mehr beiläufig möchte ich schliesslich noch folgende Daten mitteilen, die zeigen, dass auch in stark saurer Lösung Zucker eine vorübergehende Entschwellung eines frischen Muskels hervorrufen kann. Ein frischer Gastrocnemius wurde in eine Lösung von  $\frac{m}{1}$ -Traubenzucker +  $\frac{n}{1000}$ -HCl gebracht. Gewichtsveränderung:

nach 1 Std.	nach $3\frac{1}{2}$ Std.	nach 6 Std.
— 7,6%	+ 20%	+ 60%

<sup>1)</sup> Zum Vergleich führe ich hier nochmals die beim lebenden Muskel erhaltenen Gewichtsänderungen an:

	$\frac{n}{1000}$ -HCl.					
Nach	1	3	6	23	29	47 Std.
$\frac{m}{8}$ -NaCl	+ 1,0	+ 6,1	+ 9,3	+ 13,6	+ 16,3	+ 19,2
$\frac{m}{4}$ -NaCl	— 5,0	— 7,7	— 7,9	— 5,9	— 6,8	— 7,7

Sollte es möglich sein, diese Erscheinungen anders als durch osmotische Vorgänge zu erklären?<sup>1)</sup>

#### ZUSAMMENFASSUNG.

1. Gelöste Proteine beeinflussen den Wasseraustausch von Muskel und umgebender Lösung nicht in deutlich erkennbarer Weise.

2. Wird ein Muskel durch Säure unerregbar gemacht, so können osmotische Funktionen noch sehr lange nach dem Eintreten der Unerregbarkeit nachgewiesen werden (s. Diagramm). Wird ein Muskel durch Koagulation unerregbar gemacht, so verschwinden die osmotischen Eigenschaften.

<sup>1)</sup> Zur Erläuterung dieser Bemerkung muss ich noch hinzufügen, dass Martin H. Fischer in einer grossen Anzahl von Publikationen (besonders in der Kolloidchem. Zeitschr.) neuerdings behauptet, dass osmotische Vorgänge für den Wasseraustausch des Gewebes keine Rolle spielen.

## THE SEX OF A PARTHENOGENETIC TADPOLE AND FROG.

BY JACQUES LOEB AND F. W. BANCROFT.

*(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)*

THREE FIGURES.

Bataillon has shown that the unfertilized egg of the frog can be caused to develop by puncturing it. Last spring we tried the experiment in a large number of eggs of various species of anura.

The females were separated from the males, carefully washed with water and with alcohol, and then opened. The eggs were taken out of the uterus with sterilized instruments without coming in contact with the surface of the frog. About 20 per cent of the unfertilized eggs were kept as controls and 80 per cent were punctured. A few eggs were fertilized with sperm. Not a single unfertilized control egg segmented or developed. The number of unfertilized eggs which began to segment after puncture was greater in the wood frog than in the leopard frog, and amounted in the most favorable cases to about 40 per cent in the former. Only 2 of about 10,000 punctured eggs of the wood frog reached the tadpole stage, but these died before they were able to swim. The percentage of eggs of the leopard frog which reached the tadpole stage was greater. From 700 punctured eggs of the southern leopard frog, 13 good morulae were isolated the next day. On the third day, when the fertilized controls were in the gastrula stage, 13 unfertilized punctured eggs were also in the gastrula stage and 4 more eggs were developing abnormally. On the fourth day, 8 of the parthenogenetic eggs had good medullary folds and 4 had irregular folds. On the sixth day, most of the fertilized eggs hatched and 8 of the parthenogenetic eggs hatched also. Of these latter, 4 were developing regularly and 4 irregularly. Those that had not hatched were abnormal.

On the eighth day, the larvae arising from the fertilized eggs were swimming. Among the larvae arising from the unfertilized punctured eggs only 3 were normal, and their development was slightly retarded, perhaps one day. In addition, 6 parthenogenetic larvae were abnormal but still alive.

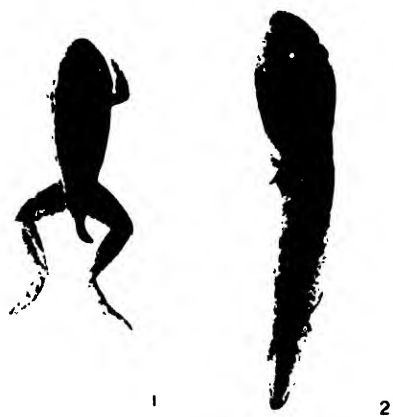
On the thirteenth day, 2 of the parthenogenetic larvae were feeding and these were the only ones which survived definitely. The other parthenogenetic larvae all died during the next few days. Of the 2 surviving larvae, one went through metamorphosis after five months. When it died, the tail was almost completely absorbed (fig. 1). Its death was probably accidental. The other lived a month longer and formed small hind legs, but died in the tadpole stage (fig. 2).

The sex glands of the frog were taken out, hardened in Tellyesnicki's fluid and sectioned; those of the tadpole were removed after it had been preserved in formalin for several months.

It was found that both parthenogenetic tadpole and frog were females (fig. 3).

Part of these experiments were made in the laboratory of the University of North Carolina, and we take pleasure in thanking Prof. H. V. Wilson for the many courtesies shown to us.

SEX OF A PARTHENOGENETIC TADPOLE.



Figs. 1 and 2 Parthenogenetic frog and parthenogenetic tadpole (natural size)



Fig. 3 Section of the ovary of the parthenogenetic frog (magnification 253)





## NEW GALVANIC PHENOMENA.

By REINHARD BEUTNER.

*(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)*

AS is well known, a galvanic cell consists in general of two different metals which are immersed in a salt solution. An arrangement of this kind will give rise to an electric current if the two metals or poles of the cell are connected by a wire. The investigation of these phenomena by Volta, after Galvani's first and famous discovery, now more than a century ago, marks the beginning of a rapid progress of our knowledge of electric phenomena.

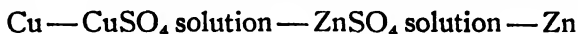
About fifty years later, it was found that organic tissues also give rise to an electric current. These currents were most extensively studied by DuBois-Reymond, who was hopeful to find new important facts concerning the physical nature of vital phenomena in general through his investigations. However, as he admitted himself, he did not succeed very well in this regard; he even failed to find a satisfactory physical explanation for the generation of electric currents by tissues. This was perhaps not so much due to an insufficiency of his physiological methods, but rather because the physics of his time did not show him how to carry out his investigations thoroughly enough to go into the details of the complicated electric phenomena in tissues. No other galvanic cells than those with metal electrodes were known to give rise to a current equal in magnitude to that of organic tissues. No metals or even metal-like substances can, however, possibly occur in tissues.

The research work done after DuBois-Reymond chiefly aimed to establish the relations between electric properties of tissues and vital phenomena. Concerning the physical nature of the biological cur-

rents, some new hypotheses were put forward, which, however, could not be proved experimentally.<sup>1</sup>

The new galvanic phenomena which I shall describe here relate to cells of a very different type from those known so far, as they are built up from water-immiscible<sup>2</sup> organic liquids and aqueous solutions. It is easy to see that an investigation with such cells may well serve to solve just those problems which DuBois-Reymond left unanswered. I can prove so far, that these new cells exhibit in a similar way some electric properties of tissues which were first observed by Dr. J. Loeb and myself last winter.

Before I explain these relations I wish to repeat first some of the more general facts about galvanic phenomena. It has been proved beyond doubt by the famous investigations of Helmholtz that the existence of a galvanic current is a thermodynamic necessity; this means that such a current must exist, if the law of the conservation of energy is true. A plain example may serve to make the matter clearer. It is known that an electric current, passing through water or certain aqueous solutions, decomposes the water into oxygen and hydrogen. The oxygen-hydrogen mixture produced represents a certain amount of energy, for it can be used either to drive a gas engine, *i. e.*, to produce mechanical work, or the energy can be transformed into heat by an explosion. This amount of energy cannot be gained from nothing; it has been taken from the current. In order to bring about the decomposition, the current, if passing through the water, has to overcome an opposing force, the so-called "polarization" or "electromotive force." In the case just discussed, the circumstance that the products of decomposition are gases and escape quickly from the electrodes intervenes. If, however, we pass a current from an outside source through a Daniell cell



it will also cause a chemical decomposition (precipitation of Zn metal

<sup>1</sup> *I. e.*, Ostwald's theory of ionic permeability of tissue membranes. Experiments of Tammann and Walden (both independent of each other) have proved that the conception of an ionic permeability is contradictory to experimental observations for artificial membranes. Haber's theory that the membranes behave like hydrogen electrodes has been proved to be contradictory to fact by Dr. J. Loeb and the writer (*Science*, 1911, vol. xxxiv, p. 886).

<sup>2</sup> *I. e.*, immiscible with water in any ratio.

from the  $\text{ZnSO}_4$  solution and solution of Cu metal, when the current passes from the Cu to the Zn), but in this case the products of decomposition are identical with the material of the electrodes, and the change brought about by the current is only a quantitative one. The current sent through this system from an outside source has therefore to overcome an opposing electromotive force in the same way. This electromotive force, however, exists also in absence of an external source and will generate a current by itself if the poles are connected.

The magnitude of a current generated by a cell depends on many unessential circumstances, like the size of the vessel which contains the cell and the resistance of the connecting wire. The electromotive force is that magnitude which is really characteristic for a definite cell arrangement, so far as the chemical composition is concerned; this electromotive force must be measured while no current is passing through the system, *i. e.*, by an electrometer, that is, an instrument the turns of which are due to electrostatic attraction.

As an electromotive force depends on a chemical reaction it is subject to the same influences as a reaction. For example, the influence of temperature upon the electromotive force has been calculated from chemical thermodynamic theories and experimentally verified.

As is well known, the concentration of a dissolved compound has a great influence upon its chemical activity. Therefore the electromotive force depends also on concentration (for instance, if we dilute the copper sulphate solution in a Daniell cell, the electromotive force decreases). Nernst, in well-known investigations on this property of electromotive force, derived a formula which states that the change of the electromotive force is proportional to the logarithm of the ionic concentration. This so-called Nernst's law, which can be easily verified by experiments, is a specialization of the thermodynamic theory of Helmholtz and of the theory of electrolytic dissociation of Arrhenius. Nernst, however, made the conception of Helmholtz more conspicuous by pointing out that the electromotive force of a cell is (mainly) composed of so-called "potential differences" at the junction of metal and solution, and that these potential differences vary regularly if the concentration is changed.

Dr. J. Loeb and the writer investigated the electromotive force of

tissues a year ago.<sup>3</sup> DuBois-Reymond and most other physiologists, who worked along this line, had used animal tissues as physiological objects, mostly frog muscles. Dr. Loeb, on the contrary, suggested the use of parts of plants for the reason that they possess a far greater chemical constancy. This was of great importance for our investigations, as it was found possible with plants only to study the very remarkable electromotive properties of tissues, while the electromotive force of animal tissues is so inconstant owing to chemical decomposition that an accurate measurement and application of physical laws appeared impossible.

It was found in this investigation<sup>4</sup> that the electromotive force of a cell arrangement which contains a piece of plant tissue as a "middle conductor"<sup>5</sup> exhibits regular and reversible changes, if the concentration of a solution in contact with the skin of the plant is varied. The magnitude of the change was about such as could be expected from Nernst's formula. This proves that the change of the electromotive force must be located at the junction of the plant skin and the aqueous solution of varying concentration.<sup>6</sup> This junction is the seat of a potential difference in the definition of Nernst, and behaves in fact similarly as the junction of a metal and a solution. There is, however, a different behavior inasmuch as with metals only a solution of a salt of the metal itself has any effect at all (*i. e.*, with silver electrodes only the concentration of a silver salt in the solution, addition of copper or zinc salts has no influence at all). The potential differences at the junction of tissues, however, vary with the concentration in the same way whether a KCl or a  $\text{CaCl}_2$  or  $\text{Na}_2\text{SO}_4$  solution is used. A solution of any (non-poisonous) electrolyte acts in the same fashion.

<sup>3</sup> Science, 1911, xxxiv, pp. 884-887.

<sup>4</sup> Concerning the experimental arrangement used, *cf. loc. cit.*

<sup>5</sup> This means that the arrangement is such that the electric conduction must take place through the tissue.

<sup>6</sup> It is known that the junction of two aqueous solutions is also the seat of a potential difference which varies with the concentration. Such so-called diffusion potentials necessarily also occur in the "cell" arrangement of Dr. Loeb and the writer. Their magnitude, however, as well as the magnitude of their variation with concentration, is by far too small to account for the change of the electromotive force observed. The same holds for the "cells" built up from water-insoluble organic substances and aqueous solutions.

An explanation of these electromotive properties of tissues can be found through the following merely physical investigations which the writer has carried out. Water-immiscible organic liquids which contain water-insoluble acids act in the same manner as the skin of plants. As an example I wish to cite here my measurements in this system:

Hg— $n/10$  KCl solution saturated with calomel—salicylic aldehyde saturated with salicylic acid—(KCl solution of varying concentration)— $n/10$  KCl solution saturated on calomel—Hg.

The electromotive force of this system (measured with Dolezalek's electrometer) varied according to the concentration of the KCl solution (in parentheses) as the following figures show.<sup>7</sup>

TABLE I.

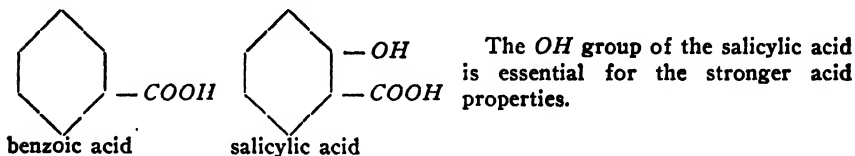
Time in minutes.	Concentration of the variable KCl solution. (In gram-molecules in a litre.)	E. m. f. observed in millivolts.	Difference of the e. m. f.'s.	Time in minutes.	Concentration of the variable KCl solution. (In gram-molecules in a litre.)	E. m. f. observed in millivolts.	Difference of the e. m. f.'s.
0	1/10	5	...	73	1/250	53	
1	1/10	6		78	1/50	25	28
3	1/50	30	24	81	1/50	24	
4	1/50	30		84	1/10	0	24
7	1/250	55	25	86	1/10	0	
9	1/250	55		88	1/2	-21	21
11	1/1250	89	34	90	1/2	-21	
12	1/1250	89		95	2 1/2	-39	18
17	1/6250	130	41	97	2 1/2	-40	
19	1/6250	130		103	1/2	-22	18
20	1/1250	89	41	104	1/2	-22	
23	1/1250	88		107	1/10	0	22
26	1/250	54	34	108	1/10	0	

These measurements, which extend over a period of nearly two hours, show to what degree of accuracy measurements of this kind may be carried out. The fact that the electromotive force of the system comes back very nearly to the same value if the same concentration is applied again proves that these measurements are far from being accidental. Comparing these electromotive forces with

<sup>7</sup> Concerning the physico-chemical technique it may be said that "liquid potentials" cannot intervene with these measurements, as the migration velocity of K' and Cl' is alike.

those observed by Dr. J. Loeb and the writer on apples,<sup>8</sup> the identical change of the electromotive forces in both cases is seen in a striking way. Instead of a KCl solution, solutions of various other salts were employed with a similar result: NaCl, NaNO<sub>3</sub>, Na-Acetate, Na<sub>2</sub>HPO<sub>4</sub>, Na<sub>2</sub>SO<sub>4</sub>, CaCl<sub>2</sub>, NH<sub>4</sub>Cl, CuSO<sub>4</sub>. These electromotive forces depend, like those of tissues, on the concentration of various salts.

Instead of the salicylic aldehyde-salicylic acid mixture various other water-immiscible organic liquids were employed. The proof that the strong acid character of the salicylic acid is responsible for these electromotive forces is found in the fact that kresol or phenol or other water-insoluble liquids of a less pronounced acid character do not show a change of electromotive force with the concentration. This is also seen from the fact that benzoic acid dissolved in benzaldehyde acted like the salicylic aldehyde-salicylic acid mixture, but the variation of potential difference is much smaller in this case; benzoic acid, in fact, is a much weaker acid than salicylic acid.<sup>9</sup>



Furthermore, I have found that water-immiscible bases exhibit a change of the potential difference in the opposite direction, as water-immiscible acid liquids, *i. e.*, aniline, toluidine, methylaniline, naphthylamine, and various other bases were studied. The electromotive force of a cell like

Hg—*m*/10 KCl solution saturated on HgCl—toluidine— (KCl solution of varying concentration) —*m*/10 KCl solution saturated on HgCl—Hg

varies in the opposite direction with the concentration of the KCl solution (in parentheses) as does the electromotive force of the arrangement described before, but to nearly the same extent and quite as regularly. Instead of KCl various other salts may also be employed with the same results.

<sup>8</sup> Compare Table I, *Loc. cit.*; Science, 1911, xxxiv, p. 885.

<sup>9</sup> This is seen from Ostwald's "Affinitätskonstanten." Also the chemical constitution of the two acids accounts for the same fact.

From water-immiscible acid and basic organic substances the new galvanic cells are built up which I have mentioned in the beginning. I wish to cite here a few-examples of such cells.

- (1)—Hg— $n/10$  KCl sol. — salic. ald. —  $m/10$  HgCl<sub>2</sub> sol. —  $n/10$  KCl sol. — Hg +  
(sat. on HgCl) (sat. on HgCl)  
electromotive force: 0.14 volt.
- (2)—Hg— $n/10$  KCl sol. — salic. ald. —  $m/10$  MgSO<sub>4</sub> —  $m/10$  KCl — Hg +  
electromotive force: 0.086 volt.
- (3)+Hg— $m/10$  KCl— $n/10$  KSCN—toluidine— $m/10$  Na<sub>2</sub>SO<sub>4</sub>— $n/10$  KCl—  
Hg—  
electromotive force: 0.25 volt.
- (4)+Hg— $n/10$  KCl — toluidine —  $m/10$  Na<sub>2</sub>HPO<sub>4</sub> —  $m/10$  KCl — Hg —  
electromotive force: 0.116 volt.
- (5)+Hg— $n/10$  KCl—toluidine— $n/1250$  KCl—salicyl. ald.— $n/10$  KCl—  
Hg—  
electromotive force: 0.19 volt.

(This last system is a new type of "double concentration cells.")

The electromotive force of some of these cells is even considerably higher than that of tissues in general.<sup>10</sup> The magnitude of the electromotive force produced by living organs, which so far seemed to be unaccountable by experiments, can no more appear as a "vital" mystery.

As was said above, the electromotive force of a galvanic cell is determined by the chemical reaction brought about by a current passing through the system. If we know the nature of this reaction for a definite cell, we have reached the most perfect explanation possible. For the new galvanic cells described above, the solution of this problem has been possible to some extent. My methods, however, to determine these reactions cannot be described in full here because this discussion involves too many questions of a special physical nature. I wish to add only that the thorough experimental and mathematical investigation of the relation between concentration and potential difference which was discussed above, is a most important step towards the solution of this problem. My mathemat-

<sup>10</sup> "Liquid potentials" can only influence the electromotive force of these systems very slightly. The metallic Hg-electrodes do not produce even the least part of the electromotive force, as they are alike and opposite (the  $n/10$  KCl always being saturated on HgCl).



ical theory concerning this relation is based upon the physico-chemical laws of mass action and distribution, and has been verified by experiment.

The result of the theory is expressed as follows:

$$\text{potent. diff.} = \frac{RT}{nF} \ln \left( \frac{1}{2C} + \sqrt{\frac{1}{4C^2} + K + \frac{M-K}{C}} \right) \cdot \text{const}$$

In this formula  $R$  is the gas constant,  $T$  the absolute temperature,  $n$  the valency of the kation,  $F$  Faraday's equivalent, "const" an integration constant.  $C$  is the concentration,  $M$  and  $K$  are calculated through the following relations:

$$M = \frac{1}{b^2 m^2} - \frac{1}{abm}$$

where  $a$  and  $b$  are special values of  $C$ ;  $m$  is determined experimentally through this relation:

$$\text{pot. diff. } (C = b) - \text{pot. diff. } (C = a) = \frac{RT}{nF} \ln m$$

Furthermore, if  $d$  is a third arbitrary value of  $C$ ,  $m'$  is determined as

$$\text{pot. diff. } (C = d) - \text{pot. diff. } (C = a) = \frac{RT}{nF} \ln m'$$

we express  $M'$  as

$$M' = m' \left( \frac{1}{2a} + \sqrt{\frac{1}{4a^2} - M} \right)$$

Through these magnitudes  $K$  (in the main formula) is found by this relation:

$$K = \frac{1}{1 - \frac{a}{d}} \left( M'^2 - \frac{M'}{d} - \frac{Ma}{d} \right)$$

The application of the formula, *i. e.*, to the measurements cited in Table I, gives the following result:

Ratio of concentrations.	Change of the potential difference.	
	Calculated.	Observed.
	milli-volts	milli-volts
1/2 over 1/10	24	21
1/10 over 1/50	22	24
1/50 over 1/250	24	28
1/250 over 1/1250	29	34
1/1250 over 1/6250	34	41

Owing to the merely physical character of this communication, the detailed biological application of the new galvanic phenomena is not to be discussed here. It seems that at this time even the physical investigation along this line is too much in its infancy, and that more details should be known in order to make a satisfactory application possible.

When our knowledge concerning electric properties of organic substances and organic tissues will be more advanced than at present, it seems likely that electric measurements will furnish a means to solve some questions concerning the physical nature of vital phenomena. Work along this line has been done by Bernstein and others, who proposed electric theories of muscular contraction, nervous irritation, etc. On account of the relations which exist between currents and chemical reactions, it seems possible that there exist some relations between electric currents in tissues and metabolism. Theories of this kind could be formulated more distinctly if the physical nature of bio-electric currents were known more accurately.

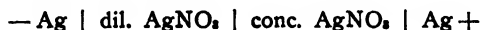
## NEW ELECTRIC PROPERTIES OF A SEMIPERMEABLE MEMBRANE OF COPPER FERROCYANIDE.

By REINHARD BEUTNER.

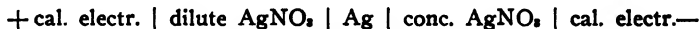
(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

I. The well-known investigations of Nernst have shown that potential differences which compose the electromotive force of whole cells are located at the junction of metal and solution and depend on the concentration of the solution according to a logarithmic law.

A concentration cell is, *e. g.*,



which may also be arranged with one single piece of Ag as a middle conductor (and calomel electrodes in order to make an electrical connection to the measuring instrument) as follows:



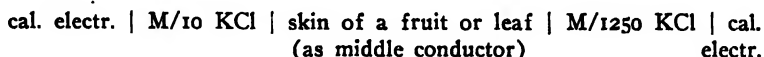
In an investigation,<sup>1</sup> based upon a thermodynamic theory of Nernst, Haber found that the middle-conducting Ag-metal in the last cell can be replaced by a solid middle-conducting layer of AgCl without causing the slightest change in the electromotive force of the cell. Through further experimental investigations, it has been proved beyond doubt that the solid AgCl possesses an electrolytic conductivity.<sup>2</sup> As to the electromotive properties, however, it resembles the metals; from a thermodynamic standpoint this peculiar property can be explained by the fact that equilibrium exists at the junction with the immiscible AgCl. A theory based upon this

<sup>1</sup> Drude's Ann., 26, 947 (1908); cf. also Nernst: Zeit. phys. Chem., 9, 140 (1892).

<sup>2</sup> This can be proved by the polarization which occurs if a current is sent through AgCl. LeBlanc's hypothesis of temporary electronic conduction in AgCl is based upon a misconception as admitted by himself (Zeit. Elektrochemie, 16, 240, 680 (1910); See Haber: Loc. cit.). Drude's Ann., 26, 972 (1908).

fact has been devised in different forms by Nernst and Haber; an account of a similar theory (based upon the same principles) was also published by the writer in an American paper.<sup>1</sup>

2. In some experimental work along biological lines by Dr. Jacques Loeb and the writer<sup>2</sup> it was found that certain vegetable and animal skins exhibited a change of potential difference with changing concentration similar to that with metals, *i. e.*, a combination:



shows an electromotive force of about 0.09 volt. Since the calomel electrodes used were filled with M/10 KCl, no liquid potential differences can occur in the whole combination (owing to the well-known fact that the migration velocities of  $\text{K}^+$  and  $\text{Cl}^-$  are equal). The electromotive force observed must therefore be located at the junctions of the aqueous solutions and skin. If we assume that the skin behaves like an electrode reversible for  $\text{K}^+$  ions and try to apply the well-known logarithmic formula of Nernst

$$E = \frac{RT}{nF} \ln \frac{C_1}{C_2}$$

(or at room temperature and for  $n=1$ )

$$\therefore 0.058 \log \frac{C_1}{C_2} = 0.12 \text{ volt}$$

we find indeed an electromotive force which about equals the electromotive force observed, at least in order of magnitude.

The sign of this electromotive force is such as one would expect with a reversibility for cations. The most characteristic property of such cells, however, is that there is not a *reversibility for  $\text{K}^+$  cations alone but for any other cation as well*, for if we replace the two KCl solutions in the above combination by two NaCl solutions of equal respective molecular concentrations, we find a similar electromotive force.

It must be said that in this case the liquid potential differences are no longer zero, yet their difference will not account for more

<sup>1</sup> Trans. Am. Electrochem. Soc., 21, 219 (1912).

<sup>2</sup> Biochem. Zeit., 41, 1 (1912).

than 10 percent of the observed electromotive force. A solution containing the salt of any alkali or alkaline earth shows a similar change of potential difference with changing concentration. Acids, caustic alkalis, and salts of the heavy metals give rise also to a small electromotive force in similar "concentration cells," but the latter have not yet been studied in detail because, in such cases, the skin is likely to be attacked chemically by the salt of the heavy metals (poisonous effect) and the phenomena are of a more complex character.

3. In this communication, I wish to show the circumstances under which the electromotive properties characteristic of the skin (or outside membrane) of tissues may *be imitated by an artificial semi-permeable membrane of copper ferrocyanide*. It is well known through the famous investigations of Traube that this artificial membrane gives rise to osmotic phenomena similar to those produced by the animal or plant membranes. Though the tissue membranes certainly possess a chemical composition which is most materially different from that of the artificial membrane, the similarity of osmotic phenomena with both membranes points to the fact that they have some identical physical properties. It is remarkable that there exists a certain similarity of electric phenomena also, as the following experiments prove.

The measurements of electromotive force with precipitation membranes were performed in the following way: A glass tube, about 2 cm wide and open at both ends, was filled with a warm solution of  $M/40 \text{ K}_4\text{Fe}(\text{CN})_6$  and 10 percent gelatine. This solution was allowed to cool down in the tube to room temperature and to become stiff. The tube was then immersed with one end in a beaker containing  $M/20 \text{ CuSO}_4$  (Fig. 1). A precipitation membrane formed instantly upon the gelatine and soon grew to considerable thickness, although hardly visible at first.

The points of two calomel electrodes were then immersed, the one into the  $M/20 \text{ CuSO}_4$  solution in the beaker, the other into a small quantity of  $M/10 \text{ KCl}$  which was poured upon the opposite (upper) end of the gelatine layer (see diagram). Electrical connections were made to a "binanten-electrometer" (of Dolezalek); the electromotive force measured was 0.115 volt and the  $\text{CuSO}_4$  solu-

tion was at the positive side; the electromotive force remained constant for 10 minutes. Another experiment of the same kind gave the electromotive force of 0.108 volt (constant); the experiment was further repeated several times with similar results, the electromotive force varying from 0.103 to 0.120 volt.<sup>1</sup>

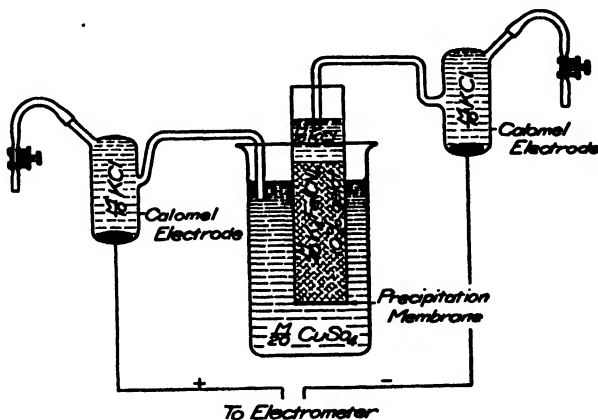
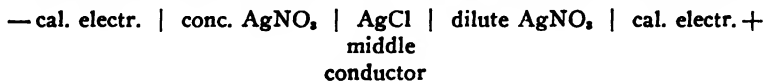


Fig. 1.

4. I wish to point out first that this electromotive force can hardly be located at any other place than on either side of the membrane. It is well known that the liquid potentials, which exist at the junction of aqueous solutions, depend on ionic mobility; their order of magnitude is up to 0.06 volt in case the solution concerned contains either  $H^+$  or  $OH^-$  or both, on account of the great ionic mobility of these two ions. As no free acid or alkali is contained in our solutions, the liquid potentials will hardly exceed 0.01 volt, *i. e.*, 10 percent of the electromotive force observed.

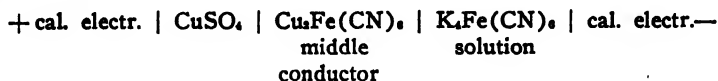
I have previously referred to Haber's research on the cell



in which the  $AgCl$  acts like  $Ag$  metal (as to the electromotive force).

<sup>1</sup> In order to measure electromotive force produced by the membrane, it is essential not to precipitate it into a porous cup as is usually done for osmotic measurements. It is known from experiments of Brunnings (Pflüger's Archiv., 117, 425 (1907)) that clay may also give rise to an electromotive force, which might interfere with the electromotive force of the membrane itself.

The question presents itself whether the insoluble  $\text{Cu}_2\text{Fe}(\text{CN})_6$  in our "cell"



acts like Cu. Apparently this is *not* the case, as is easily understood by the fact that the electromotive force of the  $\text{Cu}_2\text{Fe}(\text{CN})_6$  "cell" has the opposite sign from that of the AgCl "cell." The  $\text{CuSO}_4$  solution should be at the negative pole if the analogy of the two "cells" was right. Apparently the precipitation membrane possesses very different qualities from a solid insoluble salt (like AgCl).

How is the electromotive force observed really to be explained? I have found an answer to this question by means of experiments in which the M/20  $\text{CuSO}_4$  solution in the beaker was replaced by solutions of various composition and the influence upon the electromotive force observed.

5. The M/20  $\text{CuSO}_4$  solution was replaced,<sup>1</sup> first by more dilute  $\text{CuSO}_4$  solutions. An irregular change of the electromotive force was observed in this case, most likely on account of the fact that the membrane underwent mechanical changes through osmosis. With a more dilute  $\text{CuSO}_4$  solution in the beaker, strips of the membrane grew downward apparently on account of the greater osmotic pressure of the  $\text{K}_4\text{Fe}(\text{CN})_6$  solution upon the other side. This osmosis necessarily affects the composition of the solutions on both sides of the membranes in a way which cannot be determined or controlled experimentally.

In order to eliminate the disturbing osmotic processes further experiments were performed in such a way that the M/20  $\text{CuSO}_4$  solution was diluted *not with water but with a M/10 grape-sugar solution*, thus the osmotic pressure of the solution in the beaker was kept pretty nearly constant.<sup>2</sup>

<sup>1</sup>In replacing the solutions in the beaker care was taken not to injure the membrane.

<sup>2</sup>It is well known that a M/20  $\text{CuSO}_4$  sol. and a M/10 sugar sol. are *not* perfectly isotonic, and also the M/40  $\text{K}_4\text{Fe}(\text{CN})_6$  sol. on the opposite side of the membrane is not exactly isotonic. It was observed, however, that the differences in question did not interfere materially with the electromotive force. Irregular electromotive forces were observed only if the osmotic pressure was changed in a considerable ratio, i. e., 1:2 or 1:5.

The effect of the concentration of  $\text{CuSO}_4$  on the electromotive force was found to be such that the electromotive force decreased with decreasing concentration; the change however was very irregular and varied with the time. It can hardly be considered as an effect of concentration of the same kind as at a reversible electrode; it is more likely that irregular changes occur in the chemical composition of the membrane because one of the substances that generates the membrane is partially taken away; perhaps also a mechanical destruction of the membrane takes place.

The following observations may illustrate this irregular change of the electromotive force:

Time in minutes	Comp. of sol. in the beaker	E. m. f.
0	M/20 $\text{CuSO}_4$	0.115 volt
10	M/20 $\text{CuSO}_4$	0.115 volt
14	M/10 grape-sugar (no $\text{CuSO}_4$ )	0.077 volt
17	M/10 grape-sugar (no $\text{CuSO}_4$ )	0.070 volt
18	M/12500 $\text{CuSO}_4$ [diluted from	0.062 volt
22	M/20 $\text{CuSO}_4$ with M/10 sugar]	0.064 volt
23	M/2500 $\text{CuSO}_4$ (dil. with M/10 sugar)	0.057 volt
26	M/500 $\text{CuSO}_4$ (dil. with M/10 sugar)	0.045 volt
33	M/100 $\text{CuSO}_4$ (dil. with M/10 sugar)	0.044 volt
33	M/100 $\text{CuSO}_4$ (dil. with M/10 sugar)	0.054 volt
38	M/100 $\text{CuSO}_4$ (dil. with M/10 sugar)	0.070 volt
46	M/100 $\text{CuSO}_4$ (dil. with M/10 sugar)	0.092 volt
54	M/100 $\text{CuSO}_4$ (dil. with M/10 sugar)	0.102 volt
56	M/20 $\text{CuSO}_4$ (dil. with M/10 sugar)	0.101 volt
58	M/100 $\text{CuSO}_4$ (dil. with M/10 sugar)	0.114 volt
61	M/100 $\text{CuSO}_4$ (dil. with M/10 sugar)	0.115 volt
63	M/20 $\text{CuSO}_4$ (dil. with M/10 sugar)	0.110 volt

Further experiments gave similarly irregular data.

6. Apparently it is essential to have a constant osmotic pressure and also a constant quantity of  $\text{CuSO}_4$  in the solution in order to keep up a membrane of constant composition and properties. It was found that if these conditions were observed *the electromotive force underwent regular and reversible changes if an alkali salt was added to the solution in the beaker.* The direction of the change was such that with increasing concentration of the alkali salt the electromotive force became smaller, i. e., the solution in the beaker more negative; the effect is therefore of the same kind as with the potential differences:

tissue membrane (skin of plant, etc.)		aq. alkali salt sol. of var. concentr.
--	--	---

investigated by J. Loeb and the writer (see above).



The following experiments may serve as examples :

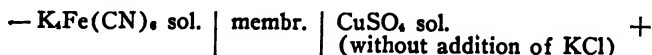
Time in minutes	Comp. of sol. in the breaker	E. m. f.	Difference of e. m. f.
0	M/40 CuSO <sub>4</sub> + M/20 sugar (no alkali salt)	0.122 volt	
17		0.123 volt	
26	M/40 CuSO <sub>4</sub> + M/40 KCl	0.020 volt	
35	M/40 CuSO <sub>4</sub> + M/40 KCl	0.019 volt	
39	M/40 CuSO <sub>4</sub> + M/200 KCl <sup>1</sup>	0.062 volt	} 0.041 volt
43	M/40 CuSO <sub>4</sub> + M/200 KCl	0.060 volt	
46	M/40 CuSO <sub>4</sub> + M/1000 KCl	0.099 volt	} 0.039 volt
54	M/40 CuSO <sub>4</sub> + M/1000 KCl	0.097 volt	
55	M/40 CuSO <sub>4</sub> + M/200 KCl	0.062 volt	} 0.035 volt
58	M/40 CuSO <sub>4</sub> + M/200 KCl	0.062 volt	
60	M/40 CuSO <sub>4</sub> + M/40 KCl	0.020 volt	} 0.042 volt
64	M/40 CuSO <sub>4</sub> + M/40 KCl	0.019 volt	
84	M/40 CuSO <sub>4</sub> + M/20 sugar (No KCl)	0.126 volt	

The change of the electromotive force is perfectly reversible and it cannot be due to a change of the "liquid potential" at the junction of the calomel electrode and the beaker. On account of the equality of migration velocity of K<sup>+</sup> and Cl<sup>-</sup> this "liquid potential" must be nearly zero and can vary but slightly with the concentration. The only other place where it can be located is at the junction of the membrane and the solution. The sense of the change is such that the *membrane behaves like an electrode reversible for K<sup>+</sup> ions*. If we try to apply Nernst's formula

$$\text{Pot. diff.} = \frac{RT}{nF} \ln c. + \text{const}$$

which holds for any reversible electrode, we find that if  $n=1$  and if  $c$  is varied in the ratio 1 : 5 (as in the above experiments), the potential difference at room temperature should vary *0.040 volt*; the values observed may be considered a good confirmation.

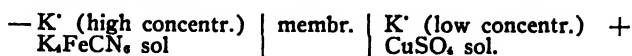
I think that from these experiments it is possible to explain why the cell



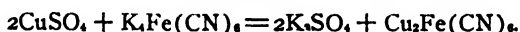
has an electromotive force as was observed. It is *due to the effect of K<sup>+</sup> ions of the K<sub>4</sub>Fe(CN)<sub>6</sub> acting on the opposite (gelatine) side*

<sup>1</sup> Diluted with M/10 grape-sugar in all following experiments even when not mentioned.

of the membrane. What we really observe is a "concentration cell" with respect to  $K^{\cdot}$ .



The  $CuSO_4$  must contain traces of  $K^{\cdot}$  salt (or  $K^{\cdot}$  ion)—even if chemically pure—on account of the fact that by the chemical reaction which forms the membrane  $K^{\cdot}$  salt is set free.



The  $K_2SO_4$  formed will be present most likely on both sides of the membrane. On the side of the  $CuSO_4$  solution in the beaker it is quickly diluted by diffusion into the great volume of the  $K^{\cdot}$  free solution. The concentration of  $K^{\cdot}$  is however not very well defined on the surface between membranes and solution (in the beaker) and this is most likely the reason why the electromotive force of the cell is rather inconstant.

7. If we stir the solution in the beaker or shake it slightly (while no  $KCl$  is added) we find a distinct but inconstant rise of the electromotive force. The following observations may serve to elucidate this fact.

*Solution in the Beaker M/40  $CuSO_4$  + M/20 Grape-Sugar.*

Time in minutes	Volt
0	0.110
10	0.116
beaker shaken 12	0.139
	electromotive force diminished, while the solution is not moved.
33	0.120
37	0.122

The effect of shaking apparently is due to an acceleration of convection, thus the  $K^{\cdot}$  concentration at and in the vicinity of the membrane becomes smaller; this must increase the electromotive force of the system if our conception is right that the "cell" really is a concentration cell for  $K^{\cdot}$ .

8. But how is the decrease of the electromotive force to be explained, which occurs if the solution is left unshaken? We can understand this from observations of a different type which relate to the *growth of the membrane*.

The membrane forms a very thin skin at the end of the first day after the system has been put together but is undergoing a constant process of growth, as is clearly seen after about two or three days, when the membrane has grown to a thickness of about 1 mm. Apparently this growth would be impossible if the membrane were perfectly impermeable for both salts,  $K_4Fe(CN)_6$  and  $CuSO_4$ , from which it is generated.<sup>1</sup>

The effect of diffusion of  $K_4Fe(CN)_6$  through the membrane seems to be as follows:

$K_4Fe(CN)_6$  will pass into the membrane at one surface and diffuse through the membrane to the other surface, where the chemical reaction with  $CuSO_4$  takes place;  $K_2SO_4$  is therefore generated at the surface of the membrane in contact with that of the  $CuSO_4$  solution. It will diffuse into this latter because of its greater solubility in water than in the membrane. All  $K_2SO_4$  formed by the reaction will be formed at the surface of the membrane if the  $CuSO_4$  does *not* diffuse through the membrane. If this is the case, the reaction between  $K_4Fe(CN)_6$  and  $CuSO_4$  must take place in the membrane itself and the  $K_2SO_4$  formed will diffuse to both surfaces. Whether the latter is true or whether  $K_4Fe(CN)_6$  *only* diffuses is unessential for our experiments. All  $K_2SO_4$  will be formed at the surface of the membrane in contact with the ferrocyanide solution, if the membrane is permeable for  $CuSO_4$  *only*, but this is not likely to be the case.

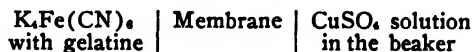
We can now understand how  $K^+$  salt is constantly being formed at the membrane; this small quantity of  $K^+$  is most likely essential for the electromotive force. I do not know however whether under certain circumstances the  $CuSO_4$  also may influence the electromotive force. If the  $K^+$  concentration is kept very low, it is possible that the cell may no longer act like a mere concentration cell for  $K^+$ , but may act like a cell, the electromotive force of which is due to a chemical reaction. Such is however not the case in all experiments communicated to this paper.

9. Another proof, for the fact that  $K^+$  ions formed through the

<sup>1</sup>Walden: *Zeit. phys. Chem.*, 10, 699 (1892) finds that a copper ferrocyanide membrane is impermeable for both  $K_4Fe(CN)_6$  and  $CuSO_4$ . This however may only mean that the permeability is small. The rather slow growth of the membrane indicates that in this sense Walden's statement is right.

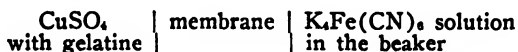
growth of the membrane determine the electromotive force, is found in the following facts: If we arrange the experiment described above in such a way that the  $\text{CuSO}_4$  solution is mixed with 10 percent gelatine and placed in the (upper) tube while the beaker contains the  $\text{K}_4\text{Fe}(\text{CN})_6$  solution (compare Fig. 1) *scarcely any electromotive force at all is observed.*

I first tried to explain this observation by means of the assumption that the gelatine may possess an action upon the potential difference like KCl, or that the commercial gelatine used may contain alkali salts as impurities. Neither assumption can be right as no action of the gelatine at all was observed, when, with the first arrangement:



some gelatine was added to the solution in the beaker.

I think it is very likely that the  $\text{K}_2\text{SO}_4$  formed through the growth of the membrane is the cause for the fact that the electromotive force of the "cell"

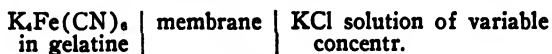


is nearly zero.<sup>1</sup> *The  $\text{K}_2\text{SO}_4$  formed in the boundary region of the  $\text{CuSO}_4$  solution is kept there in a high concentration as the convection is retarded by the gelatine.* Therefore, the concentration of  $\text{K}^+$  is nearly equal on both sides of the membrane.

I wish to add that with this cell no considerable effect upon the electromotive force can be expected through addition of further alkali salt to the  $\text{K}_4\text{Fe}(\text{CN})_6$  solution, as the  $\text{K}^+$  concentration is thus varied only slightly. This was also confirmed by experiment.

If the  $\text{K}_4\text{Fe}(\text{CN})_6$  concentration was varied, the electromotive force underwent small changes.

10. Furthermore, some measurements of the cell



were performed while *no  $\text{CuSO}_4$  was added.* For the reasons given above, the electromotive force is not exactly reversible in this case (compare page 477).

<sup>1</sup> The values observed were 0.003 to 0.010 volt.

Time in minutes	Solution in the beaker	E. m. f.	Difference
0	M/20 KCl	0.003 volt	
1	M/20 KCl	0.003 volt	0.038 volt
7	M/100 KCl	0.041 volt	
10	M/100 KCl	0.039 volt	
12	M/500 KCl	0.076 volt	0.037 volt
14	M/500 KCl	0.076 volt	
16	M/2500 KCl	0.101 volt	
19	M/500 KCl	0.072 volt	0.029 volt
22	M/500 KCl	0.071 volt	0.033 volt
23	M/100 KCl	0.038 volt	
24	M/100 KCl	0.037 volt	
30	M/20 KCl	0.003 volt	0.034 volt

11. As was said above (compare page 473), the membrane is not reversible for  $K^+$  ions only, but for other ions in the same way. In other words, the electromotive force is changed in the same way if  $NaCl$  or  $Na_2SO_4$  be added to the  $CuSO_4$  solution in the beaker instead of  $KCl$ , as the following measurements show:

Time in minutes	Comp. solution in the beaker	E. m. f.	Difference of e. m. f.
0	M/40 $CuSO_4$	0.125 volt	
2	M/40 $CuSO_4$ + 1/80 $Na_2SO_4$	0.035 volt	
7	M/40 $CuSO_4$ + 1/80 $Na_2SO_4$	0.028 volt	
11	M/40 $CuSO_4$ + 1/80 $Na_2SO_4$	0.031 volt	
15	M/40 $CuSO_4$ + 1/40 $NaCl$	0.032 volt	
21	M/40 $CuSO_4$ + 1/40 $NaCl$	0.030 volt	
26	M/40 $CuSO_4$ + 1/40 $NaCl$	0.028 volt	0.051 volt
31	M/40 $CuSO_4$ + 1/40 $NaCl$	0.079 volt	
34	M/40 $CuSO_4$ + 1/400 $NaCl$	0.080 volt	
36	M/40 $CuSO_4$ + 1/400 $NaCl$	0.080 volt	0.0515 volt
46	M/40 $CuSO_4$ + 1/40 $NaCl$	0.0285 volt	
48	M/40 $CuSO_4$ + 1/40 $NaCl$	0.0285 volt	

The fact that  $Na_2SO_4$  and  $NaCl$  influence the electromotive force in exactly the same way shows that the  $Cl^-$  ion of  $NaCl$  or  $KCl$  does not have any specific action. The change of the electromotive force for a tenfold dilution should be 0.058 volt according to Nernst's formula.

The following measurements relate to  $NH_4Cl$ :

Time in minutes	Comp. of solution in the beaker	E. m. f.	Difference of e. m. f.
0	M/40 $CuSO_4$	0.105 volt	
3	M/40 $CuSO_4$ + 1/40 $NH_4Cl$	0.024 volt	0.053 volt (calc. 0.05)
5	M/40 $CuSO_4$ + 1/40 $NH_4Cl$	0.021 volt	
7	M/40 $CuSO_4$ + 1/400 $NH_4Cl$	0.074 volt	
9	M/40 $CuSO_4$ + 1/400 $NH_4Cl$	0.073 volt	0.053 volt
10	M/40 $CuSO_4$ + 1/40 $NH_4Cl$	0.021 volt	
10	M/40 $CuSO_4$ + 1/40 $NH_4Cl$	0.020 volt	
27	M/40 $CuSO_4$	0.109 volt	

HCl also had a distinct influence on the electromotive force, as the following experiments prove:

Time in minutes	Comp. of solution in the beaker	E. m. f.	Difference of e. m. f.
0	M/40 CuSO <sub>4</sub> + 1/40 NaCl	+0.029 volt	
12	M/40 CuSO <sub>4</sub> + 1/40 HCl	-0.035 volt	(solution at negative pole)
19	M/40 CuSO <sub>4</sub> + 1/40 HCl	-0.004 volt	0.046 volt
20	M/40 CuSO <sub>4</sub> + 1/400 HCl	+0.042 volt	
29	M/40 CuSO <sub>4</sub> + 1/400 HCl	+0.041 volt	0.037 volt
37	M/40 CuSO <sub>4</sub> + 1/400 HCl	+0.043 volt	
39	M/40 CuSO <sub>4</sub> + 1/4000 HCl	+0.080 volt	0.036 volt
40	M/40 CuSO <sub>4</sub> + 1/4000 HCl	+0.079 volt	
42	M/40 CuSO <sub>4</sub> + 1/400 HCl	0.043 volt	0.044 volt
48	M/40 CuSO <sub>4</sub> + 1/400 HCl	0.042 volt	
48	M/40 CuSO <sub>4</sub> + 1/40 HCl	-0.002 volt	
50	M/40 CuSO <sub>4</sub> + 1/40 HCl	-0.003 volt	

The quantitative application of Nernst's formula seems to be less satisfactory in the last measurements (relating to HCl), the value calculated (0.058 volt) differing considerably from those observed. It must be said, however, that in this case the "liquid potential" at the junction of the left (see Fig. 1) electrode is neither zero nor practically independent of the HCl concentration on account of the great migration velocity of H<sup>+</sup> ions.

The sign of the "liquid potential" is opposed in this case to the sign of the potential difference at the membrane; this may account for the observation of too small differences as compared with the theoretical value. A quantitative calculation, however, seems impossible for the reason that the considerable amount of sugar present in some solutions is not without influence upon "liquid potentials."<sup>1</sup>

*Bivalent cations seem to have no influence upon the electromotive force* if added to the solution in the beaker, as the following measurements show.

As to bivalent cations the artificial membrane seems to behave distinctly differently from tissue membrane, at least so far as observed by Dr. Loeb and the writer. It was found<sup>2</sup> that the potential

<sup>1</sup> This fact is not accounted for by the well-known theory of "liquid potentials" by Planck and Nernst. By experiments of Oker-Blom (Pflüger's Archiv., 84, 191), however, an influence of non-electrolytes upon liquid potentials is clearly demonstrated.

<sup>2</sup> Loc. cit.

	Solution	E. m. f.
I	M/40 CuSO <sub>4</sub>	0.123 volt
II Time in minutes	M/40 CuSO <sub>4</sub> + 1/40 CaCl <sub>2</sub>	0.130 volt <sup>1</sup>
0	M/40 CuSO <sub>4</sub>	0.109 volt
2	M/40 CuSO <sub>4</sub> + 1/40 MgCl <sub>2</sub>	0.122 volt <sup>2</sup>
3	M/40 CuSO <sub>4</sub> + 1/40 MgCl <sub>2</sub>	0.119 volt
4	M/40 CuSO <sub>4</sub> + 1/40 MgCl <sub>2</sub>	0.118 volt
5	M/40 CuSO <sub>4</sub> + 1/400 MgCl <sub>2</sub>	0.128 volt
6	M/40 CuSO <sub>4</sub> + 1/400 MgCl <sub>2</sub>	0.126 volt
7	M/40 CuSO <sub>4</sub>	0.126 volt

differences at the skin of plants are influenced by CaCl<sub>2</sub> solutions nearly in the same way as by KCl or NaCl solutions.

12. The observations described prove that an artificial membrane acts like an electrode, reversible for monovalent ions of different kinds in a similar way as a tissue membrane acts. As to the further question, as to what circumstances may possibly bring about this electromotive effect, I think that the same explanation can be given here as for potential differences at tissue membrane. For the latter I have proved in a mathematical paper<sup>3</sup> that the following relation holds:

$$\text{Pot. diff.} = 0.058 (\log C - \log \{1 + \sqrt{1 + 10^8 m^2 C^2}\}) + \text{const.})$$

where pot. diff. is expressed in volts at room temperature,  $C$  is the concentration of the aqueous solution, and  $m$  is given by the following relation:

$$0.058 \log 1/m = \text{limiting value of the pot. diff. pot. diff. for } C = n/500.$$

The potential difference in fact reaches a limiting value at high concentrations in this case, *i. e.*, the electromotive force of a cell:

Concentrated solution | tissue membrane | dilute solution

becomes zero, if the concentration of the (more) dilute solution is higher than a certain value. The formula was tested experimentally by means of measurements on apples and was found to agree well with the observations.

No limiting value of the potential difference was observed with the potential differences investigated in this paper, but this may be

<sup>1</sup> This small rise of the electromotive force is not reversible and most likely more or less accidental.

<sup>2</sup> See footnote 1, page 483.

<sup>3</sup> Presented at the Eighth International Congress of Applied Chemistry in New York, Vol. 22, 29 (1912). A communication of the same paper is also found in the *Biochem. Zeitschrift*, 47, 73 (1912).

due to the fact that the concentration could not be increased high enough to reach the limiting value. I intend to investigate this point further.

The derivation of the formula cited above was based primarily upon the fact that the junction between any two phases behaves like an electrode, *i. e.*, that the potential difference located there can be expressed as

$$\frac{RT}{nF} \cdot \ln \frac{c_1}{c_2} \cdot \text{const.}$$

where  $c_1$  is the concentration of an ion common to both phases in the one phase,  $c_2$  the same in other place.<sup>1</sup> Furthermore, the following assumptions were made:

a. That a complete reaction takes place between a constituent of the membrane and the aqueous solution. In this reaction a salt is formed which is insoluble in water but distributed in the membrane homogeneously.

b. That the electrolyte as such is contained in the membrane in small concentration and that in accordance with the law of partition the ratio of concentration of electrolyte in membrane and in water is constant. The details of the derivation will be found in the cited mathematical paper.

The main point proved by the agreement of the observations with this mathematical theory is *that the membrane contains a water-insoluble electrolyte of the same cation for which it is reversible*. I am inclined to believe that with the copper ferrocyanide membrane this water-insoluble electrolyte in the membrane is a complex compound of copper ferrocyanide and alkali ferrocyanide, *i. e.*, the membrane is not copper ferrocyanide as indicated by the formula  $\text{Cu}_2\text{Fe}(\text{CN})_6$  but contains alkali salt also.

This alkali salt is Na, K, or  $\text{NH}_4$  salt according to the composition of the aqueous solution; this is possible on account of a chemical reaction which takes place between the alkali salt of the membrane, *i. e.*,  $\text{KX}$  and the alkali salt of the solution, *i. e.*,  $\text{NH}_4\text{Cl}$ , whereby  $\text{NH}_4\text{X}$  (in the membrane) and  $\text{KCl}$  is formed.

13. The theory explained seems to be very hypothetical on ac-

<sup>1</sup> Concerning the derivation of this formula (which was first derived for a special case by Nernst), I refer to my paper, Trans. Am. Electrochem. Soc., 21, 219 (1912).



count of the fact that neither the exact chemical constitution nor the physical properties of the membrane are known to a satisfactory extent. Further evidence of the real nature of these new electric phenomena is however gained through *the new galvanic phenomena with water-insoluble organic liquids which the author first observed recently*. It was found that water-insoluble liquids of an acid character exhibit an electromotive force like the membranes described; water-insoluble bases, however, exhibit a change of the potential difference in the opposite direction.

With experiments of this kind the composition of the second phase (*i. e.*, the water-insoluble liquid which replaces the membrane in our experiments) *was* known, as well as some of its physical properties. In a great number of experiments, in which both the composition of the second phase as well as the composition of the aqueous solution was varied, the assumptions described above were found to be correct; the physical conditions which cause potential differences reversible for more than one ion, are mainly due to the formation of a water-insoluble compound by a chemical reaction between the aqueous electrolyte and a constituent of the second phase.<sup>1</sup>

I think that a study of membranes of various compositions, which I intend to perform, will give further evidence concerning these questions.

#### CONCLUSIONS.

The main results of the experiments described are:

1. A precipitation membrane of copper ferrocyanide behaves like an electrode, reversible for monovalent positive ions of different kinds.
2. The electromotive force of the system  $K_4Fe(CN)_6$  aq. sol.—precipitation membrane— $CuSO_4$  aq. sol. is due to a concentration cell with  $K^+$  ions. The  $K^+$  salt concentration of the  $CuSO_4$  solution is due to  $K_2SO_4$ , which is generated by the formation and steady increase in thickness of the membrane. The  $K^+$  concentration in the boundary region of the  $CuSO_4$  solution, which is essential for the electromotive force, depends mainly on the velocity of diffusion into that part of the  $CuSO_4$  solution which is free from  $K_2SO_4$ .

<sup>1</sup> As to further details of this investigation, I refer to a publication which is soon to appear in the Transactions of the Am. Electrochem. Soc.

## THE RELATIVE INFLUENCE OF WEAK AND STRONG BASES UPON THE RATE OF OXIDATIONS IN THE UNFERTILIZED EGG OF THE SEA URCHIN.\*

BY JACQUES LOEB AND HARDOLPH WASTENEYS.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

1. About six years ago it was shown by Loeb that bases (NaOH, KOH) can induce artificial parthenogenesis in the eggs of sea urchins and annelids.<sup>1</sup> This action of the bases was suppressed or retarded when the oxidations in the egg were suppressed or retarded by the withdrawal of oxygen from the alkaline solution or by the addition of a small amount of KCN.<sup>2</sup> He therefore concluded that the bases induced artificial parthenogenesis through an acceleration of the rate of oxidations in the egg. Last summer the same author showed that the weak base  $\text{NH}_4\text{OH}$  is much more efficient for the causation of artificial parthenogenesis than the strong bases NaOH, KOH or tetraethylammoniumhydroxide.<sup>3</sup> This he explained on the basis of the fact found by O. Warburg,<sup>4</sup> and extended by Harvey,<sup>5</sup> that the weak bases diffuse more readily into the egg while the strong bases do not. This behavior was an analogue to the fact found by Loeb in 1905 that weak acids like  $\text{CO}_2$  or the monobasic fatty acids induce membrane formation and development readily in the unfertilized egg of the sea urchin, while the strong acids like HCl or oxalic acid are very ineffective.<sup>6</sup> This

\* Received for publication, March 15, 1913.

<sup>1</sup> Loeb: *Pflüger's Archiv*, cxviii, p. 572, 1907.

<sup>2</sup> Loeb: *ibid.*, cxviii, p. 30, 1907.

<sup>3</sup> Loeb: *Journ. of Exp. Zoölogy*, xiii, p. 577, 1912.

<sup>4</sup> Warburg: *Zeitschr. f. physiol. Chem.*, lxvi, p. 305, 1910.

<sup>5</sup> Harvey: *Journ. of Exp. Zoölogy*, x, p. 507, 1911.

<sup>6</sup> Loeb: *Univ. of Calif. Publ. Physiol.*, ii, p. 113, 1905; *Biochem. Zeitschr.*, xv, p. 254, 1909.

fact he explained on the assumption that the weak acids diffuse readily into the egg while the strong acids do not. While the bases only acted in the presence of oxygen, the action of the acids was independent of oxidations in the egg.

2. More recently Loeb extended his investigations on the relative efficiency of weak and strong bases for artificial parthenogenesis to a larger number of bases. The egg used was that of an annelid, *Polynoe*. It was found that the bases, in regard to their efficiency for this purpose, may be divided into three groups. The most efficient were the amines of which benzyl, butyl, ethyl and methylamine were tried. Benzyl and butylamine were possibly a little more efficient than ethyl and methylamine. Next in efficiency were  $\text{NH}_4\text{OH}$  and trimethylamine. The least efficient were the strong bases  $\text{NaOH}$  and tetraethylammoniumhydroxide.

The relative efficiency of these bases for the causation of artificial parthenogenesis was tested in this way that the unfertilized eggs of *Polynoe* were put into solutions containing the same molecular concentration of these various bases. The reciprocal value of the time required for the various bases to cause development was the measure of their relative efficiency. The simple amines acted most quickly, then followed  $\text{NH}_4\text{OH}$  and trimethylamine; the strong bases required more time than either of the two other groups of bases.

3. These experiences suggested an investigation of the influence of the various bases upon the rate of oxidations in the unfertilized egg, to find out whether the weaker bases raised the rate of oxidations more than the stronger bases.

The experiments were carried out on the unfertilized egg of *Strongylocentrotus purpuratus* in Pacific Grove, California. The oxygen consumption was determined according to Winkler's methods. The experiments were made in this way that the oxygen consumption for the same lot of eggs was first determined in a neutral solution and then for the same length of time and the same temperature in an alkaline solution. The experiments were made in a half gram molecular mixture of  $\text{NaCl} + \text{KCl} + \text{CaCl}_2$  in that proportion in which these three salts are contained in the sea water.

The reader who is interested in the details of the method may be referred to our former publications or to those of Warburg.<sup>7</sup>

We first give the results of a series of experiments in which the relative influence of various bases was compared. The time of exposure was one hour and twenty-five minutes; the temperature, 18° C. The concentration of the bases chosen was that found most effective in Loeb's previous experiments on artificial parthenogenesis. The oxygen consumption was first measured in a neutral solution and then for the same eggs in the alkaline solution in which 0.3 cc. of  $\frac{N}{10}$  of the various bases was added to 50 cc. of the solution.

TABLE I.

NUMBER OF EXPERIMENT	NATURE OF SOLUTION	OXYGEN CONSUMED	ACCELERATION OF RATE OF OXIDATION BY THE BASE
		<i>mgm.</i>	
I	{ Neutral..... 50 cc. neutral +0.3 cc. $\frac{N}{10}$ NaOH.....	0.28 0.40	1.43
II	{ Neutral..... 50 cc. neutral +0.3 cc. $\frac{N}{10}$ tetraethylammoniumhydroxide.....	0.15 0.22	1.50
III	{ Neutral..... 50 cc. neutral +0.3 cc. $\frac{N}{10}$ NH <sub>4</sub> OH.....	0.30 0.81	2.70
IV	{ Neutral..... 50 cc. neutral +0.3 cc. $\frac{N}{10}$ trimethylamine....	0.40 1.19	3.00
V	{ Neutral..... 50 cc. neutral +0.3 cc. $\frac{N}{10}$ methylamine.....	0.25 1.18	4.70
VI	{ Neutral..... 50 cc. neutral +0.3 cc. $\frac{N}{10}$ ethylamine.....	0.28 1.35	4.80
VII	{ Neutral..... 50 cc. neutral +0.3 cc. $\frac{N}{10}$ butylamine.....	0.32 1.23	3.80
VIII	{ Neutral..... 50 cc. neutral +0.3 cc. $\frac{N}{10}$ benzylamine....	0.22 1.30	5.90

These experiments, which were repeated with the same result, show clearly that the relative efficiency of the bases for inducing

<sup>7</sup> Loeb and Wasteneys: *Biochem. Zeitschr.*, xxviii, p. 340, 1910; Warburg: *loc. cit.*

artificial parthenogenesis in the unfertilized eggs of *Polynoe* and the sea urchin runs parallel with their accelerating influence upon the rate of oxidations in the unfertilized egg of the sea urchin. Incidentally it may be stated that  $\text{NaHCO}_3$  does not accelerate the rate of oxidations in the unfertilized egg nor does it cause artificial parthenogenesis.

4. We compared next the relative effect of various concentrations of  $\text{NaOH}$  and  $\text{NH}_4\text{OH}$  upon the rate of oxidations in the unfertilized sea urchin egg, during one hour. We will state only the coefficient of the rate of oxidation in the various solutions, calling the rate in the neutral solution 1.

TABLE II.

AMOUNT OF BASE ADDED TO 50 cc. $\frac{N}{10}$ ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ )	COEFFICIENT OF ACCELERATION OF OXIDATIONS IN	
	$\text{NaOH}$	$\text{NH}_4\text{OH}$
0.2 cc. $\frac{N}{10}$	1.35	1.29
0.5 cc. $\frac{N}{10}$	1.87	5.00
0.8 cc. $\frac{N}{10}$	1.74	5.94
1.0 cc. $\frac{N}{10}$	2.06	6.10
1.4 cc. $\frac{N}{10}$	2.32	6.40
2.0 cc. $\frac{N}{10}$	3.42	6.23
2.5 cc. $\frac{N}{10}$	4.57	5.70
3.0 cc. $\frac{N}{10}$	7.60	6.00

The reader will notice the striking difference in the behavior of  $\text{NH}_4\text{OH}$  and  $\text{NaOH}$ . Very low concentrations of  $\text{NH}_4\text{OH}$  (0.5 cc. per 50 cc. of solution) raise the rate of oxidations in the fertilized egg almost to the maximal height, and a further rise in the concentration has only a slight effect upon the rate of oxidation. Low concentrations of  $\text{NaOH}$  raise the rate of oxidation only little and its efficiency rises steadily with an increase in its concentration. We could not go beyond the concentrations used in this experiment since the addition of 3 cc. of  $\frac{N}{10}$   $\text{NaOH}$  to 50 cc. of  $\text{NaCl} + \text{KCl} + \text{CaCl}_2$  leads already to a cytolysis of the eggs.

It is also of interest to point out that in the eggs of *Strongylocentrotus purpuratus* fertilization by sperm raises the rate of oxi-

dation to about six times that in the unfertilized eggs. This seems to indicate that with  $\text{NH}_4\text{OH}$  it is not possible to raise the rate of oxidations in the unfertilized egg beyond the limit to which it can be raised by the fertilization with sperm. It is not possible to decide whether the same holds true for  $\text{NaOH}$ .

The fact that  $\text{NH}_4\text{OH}$  reaches its maximum effect at so low a concentration is not confined to  $\text{NH}_4\text{OH}$  but is also shared by the amines, as the following table shows.  $\text{NH}_4\text{OH}$  and ethylamine were compared.

TABLE III.

AMOUNT OF BASE ADDED TO 50 cc. $\frac{N}{2}$ ( $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ )	COEFFICIENT OF ACCELERATION OF OXIDATIONS IN	
	$\text{NH}_4\text{OH}$	Ethylamine
0.1 cc. $\frac{N}{10}$	1.9	1.4
0.2 cc. $\frac{N}{10}$	2.9	3.0
0.4 cc. $\frac{N}{10}$	3.4	4.3
0.8 cc. $\frac{N}{10}$	3.9	4.2

Ethylamine reaches its maximal efficiency at the concentration of 0.4 cc. of  $\frac{N}{10}$  base to 50 cc. of the neutral liquid; and for  $\text{NH}_4\text{OH}$  the limit is nearly at the same point as in our previous experiment.

5. It seems natural to connect this difference in the behavior of  $\text{NaOH}$  and  $\text{NH}_4\text{OH}$  with the difference in the rate of their diffusion into the unfertilized egg. If the rate of diffusion of  $\text{NaOH}$  is extremely slow and that of  $\text{NH}_4\text{OH}$  fast, it is natural that the maximal rate of oxidation should be reached with a lower concentration of  $\text{NH}_4\text{OH}$  than of  $\text{NaOH}$ . We determined the consumption of oxygen for the same lot of eggs for eight consecutive hours in 50 cc. of sea water + 1.0 cc. of  $\frac{N}{10}$   $\text{NaOH}$ . The following table gives the result.

This table shows that the longer the  $\text{NaOH}$  acts upon the egg the higher the amount of oxygen becomes which is consumed per hour. This would agree with the assumption that the  $\text{NaOH}$  diffuses slowly into the egg and that the increase in the rate of oxidations in the unfertilized egg is determined by the amount of base which has diffused into the egg.

TABLE IV.

*Consumption of Oxygen at 18° in 50 Cc. of Normal Sea Water + 1.0 Cc. of  $\frac{N}{10}$  NaOH in Eight Consecutive Hours.*

	OXYGEN CONSUMED	COEFFICIENT OF OXIDATION
	<i>mgm.</i>	
1st hour . . . . .	0.24	1.00
2d hour . . . . .	0.38	1.57
3d hour . . . . .	0.45	1.87
4th hour . . . . .	0.50	2.08
5th hour . . . . .	0.58	2.42
6th hour . . . . .	0.72	3.00
7th hour . . . . .	0.92	3.84
8th hour . . . . .	0.95	3.96

6. It was to be expected that since  $\text{NH}_4\text{OH}$  is very soluble in the egg, *i. e.*, diffuses rapidly into the egg, its maximum effect would be reached during the first hour. This was found to be true, as the following table shows.

TABLE V.

*Consumption of Oxygen by Unfertilized Eggs at 18° in 50 Cc. of Normal Sea Water + 0.8 Cc. of  $\frac{N}{10}$   $\text{NH}_4\text{OH}$ .*

		OXYGEN CONSUMED	COEFFICIENT OF OXIDATION
		<i>mgm.</i>	
Normal sea water . . . . .		0.15	1.0
50 cc. sea water + 0.8 cc. $\frac{N}{10}$ $\text{NH}_4\text{OH}$ . . . . .	1st hour	0.99	6.7
50 cc. sea water + 0.8 cc. $\frac{N}{10}$ $\text{NH}_4\text{OH}$ . . . . .	2d hour	1.03	6.9
50 cc. sea water + 0.8 cc. $\frac{N}{10}$ $\text{NH}_4\text{OH}$ . . . . .	3d hour	0.87	5.8
50 cc. sea water + 0.8 cc. $\frac{N}{10}$ $\text{NH}_4\text{OH}$ . . . . .	4th hour	0.86	5.7
50 cc. sea water + 0.8 cc. $\frac{N}{10}$ $\text{NH}_4\text{OH}$ . . . . .	5th hour	0.83	5.5

7. We intend to investigate in future experiments whether these effects of bases upon the rate of oxidations in the unfertilized eggs are irreversible, *i. e.*, will continue if the eggs are put into normal sea water after the treatment with alkali. But, we have an experiment which possibly serves the same purpose. We measured the amount of oxygen consumed in one hour by the eggs mentioned in the last table in the same solution sixteen and twenty-four hours, respectively, after the experiment. In the meantime the eggs had been kept at a low temperature in normal sea water. The rate of

oxidation after sixteen or twenty-four hours was practically the same as in the second hour.

8. These experiments prove two facts, first, that the weaker bases increase the rate of oxidations in the unfertilized egg more than the stronger bases; and second, that this difference is due to the fact that the weaker bases diffuse more rapidly into the egg than the strong bases.

The connection between the oxidative action of bases and artificial parthenogenesis lies in the fact that the essential factor in artificial parthenogenesis is an alteration of the surface or cortical layer of the egg which results in a membrane formation. Loeb has shown in former experiments that bases cause the swelling and liquefaction of the gelatinous mass (the so-called chorion) which surrounds the immature egg of a mollusc, *Lottia*, and that this action of bases is inhibited by lack of oxygen and by the addition of KCN.<sup>8</sup> This year the same author convinced himself that weak bases like the amines and  $\text{NH}_4\text{OH}$  bring about the dissolution of the chorion much more rapidly than the strong bases  $\text{NaOH}$  and tetraethylammoniumhydroxide. It is possible that the induction of artificial parthenogenesis in the sea urchin egg by bases depends upon the occurrence of a similar process in the cortical layer of this egg.<sup>9</sup> We may imagine that they act by accelerating the rate of oxidation of a substance (existing in the cortical layer of the egg?) whereby the membrane formation and consequently the development of the egg is induced.

#### SUMMARY.

The paper shows that the weak bases which are more efficient in causing artificial parthenogenesis are also more efficient in raising the rate of oxidations in the unfertilized egg. This lends further support to the view expressed by Loeb that the bases cause artificial parthenogenesis through an acceleration of the rate of oxidations.

The experiments were carried on at the Herzstein Laboratory in Monterey, California, and the authors express their thanks to Drs. Robertson, Maxwell and Moore for their kind hospitality.

<sup>8</sup> Loeb: *Univ. of Calif. Publ. Physiol.*, iii, p. 1, 1905.

<sup>9</sup> Loeb: *Die chemische Entwicklungserregung des tierischen Eies*, Berlin, 1909, p. 181.



## ON THE DIFFERENCES IN THE EFFECTS OF STIMULATION OF THE TWO VAGUS NERVES ON RATE AND CONDUCTION OF THE DOG'S HEART.\*

By ALFRED E. COHN, M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)*

PLATES 69-76.

### INTRODUCTION AND HISTORICAL REVIEW.

In 1869 Meyer (1) first observed differences between the right and left vagus nerves of the tortoise as regards their action on the heart. Since that time many investigations concerning the function of the vagus nerves have been made, but the differences between the action of the two nerves have not been emphasized and have not been reported in detail.

Garrey (2), Robinson and Draper (3), and the present writer (4) only have considered that in addition to quantitative differences obtained on stimulation, essential or qualitative differences in the function of the two nerves may exist, and have studied the subject of vagus function from this point of view.

In the present communication the extensive literature dealing with the question of vagus function is reviewed in order to indicate what observations relating to differences in the action of the two vagi have already been noted. Observations from which some comparison between the action of the right and left vagus nerves may be drawn have been made on a large number of animals. In fishes and amphibia the only differences that have been noted have been differences in degree; stimulation of one nerve has been said to be more effective than stimulation of the other. Gaskell noted that in frogs stimulation of the left vagus caused blocking, but he did not stimulate the right nerve and does not describe any difference between the two nerves as regards the production of this phenomenon.

In reptiles (snake, tortoise, and crocodile) a greater effect on the

\* Received for publication, June 18, 1912.

heart rate has been found to follow stimulation of the right nerve than stimulation of the left. Gaskell suggested a qualitative difference between the two nerves and stated that the "nerve fibers supplying those muscular fibers of the sinus, which by their automatic contractions originate the rhythmical beats of the heart, run almost exclusively in the right vagus." Unfortunately he did not assign any definite functions to the left vagus, and drew no definite conclusions concerning differences in function between the two nerves. Garrey, who described differences between the two nerves, ascribed to each a homolateral distribution and function.<sup>1</sup> He failed to note, however, whether stimulation of one or the other vagus produced any effect on the atrioventricular bundle, similar to the effect produced by the stimulation of the left vagus nerve in dogs as described in the present communication. In birds no constant differences between the action of the two vagus nerves have been described. Only in mammals have there been observed phenomena resulting from stimulation of one or the other of the vagus nerves, which may be interpreted as definitely indicating functional differences between them. McWilliam (5), François-Franck (6), Roy and Adami (7), Bayliss and Starling (8), Rehfisch (9), Hering (10), and Rothberger and Winterberg (11, 12) have all published curves or have described phenomena occurring in their experiments which indicate such differences and which may readily be correlated with the observations made by us. In some instances the unusual effects noted by these writers followed stimulation of the left vagus nerves; in other instances the nerve stimulated is not mentioned. None of these writers, however, have made a careful study of the exact nature of the differences in function of the two nerves.

The following is a more detailed review of the literature concerning these various observations.<sup>1</sup>

In studying the action of the vagus in eels McWilliam (13) found that "stimulation of either the right or the left vagus is effective. And when prolonged excitation of one nerve fails to keep up the cardiac inhibition any longer, stimulation of the other vagus usually causes a further arrest of the heart's

<sup>1</sup> A complete bibliography and review have not been attempted. Omissions have been made, but it is believed that the present state of knowledge has been shown.

action."<sup>3</sup> He considered that "the ventricle is not directly influenced by stimulation of the vagus nerve," since it may be arrested but still remain quite irritable.<sup>4</sup>

The vagus nerves in frogs have been studied by Gaskell (14), Hofmann (15), and Guyénot (16). In Gaskell's experiments the left vagus nerve was invariably stimulated. He found that following vagus stimulation the ventricles "beat for a time synchronously with every second auricular beat." In other words, he obtained an incomplete auriculoventricular dissociation. Hofmann (15) found that "the difference between the right and left vagi in the same animal consists in the fact that stimulation of the right vagus often results in the arrest of the heart, while stimulation of the left vagus, in only a weakening of the contraction."<sup>5</sup> These conclusions differ from those of Gaskell in that the latter considered that the left vagus has mainly a dromotropic function, while Hofmann considered the inotropic function of the nerve to be most important. Guyénot (16), while working on *Rana temporaria*, *Rana esculenta*, and on *Bufo vulgaris*, describes the action of the two nerves as follows: He says that (a) "in a certain number of cases the right vagus stopped the heart, while the left increased diastolic dilatation and diminished the force of the contractions without changing the number of pulsations. At other times the left was inactive, while the right did not alter the rate but increased diastolic dilatation, and left the systoles unchanged. The amplitude of the beats was increased." (b) "Sometimes stimulation of one or the other vagus nerve modified neither the number nor the force of the heart beats." (c) Stimulation of the vagus nerve often had only an accelerator or hypertonic action. McWilliam (17) studied the vagi in newts and found them somewhat different from those in frogs. For he says, "Either vagus can arrest the whole heart, but the right vagus acts much more readily and powerfully upon the ventricle than the left vagus does." The effect of the left vagus on the ventricle was not merely due to blocking, for the ventricular muscle itself had lost its irritability.

In a tortoise (*Emys lutaria*), Meyer (1) was able to produce no changes in the pulsations of the heart by stimulating the left vagus, although he could arrest the contractions of the heart with a current of the same strength applied to the right vagus. In two small specimens from Venice he also found differences between the two nerves; for stimulation of the left nerve in them slowed but did not stop the heart. Later observations on the turtle's heart were made by Gaskell (18, 19), Mills (20, 21), Kazem-Beck (22), Garrey (2), Kronecker (23), and Guyénot (24). The findings of Gaskell agreed with those of Meyer, for left vagus stimulation produced a marked alteration in force of the auricular contractions, the effect sometimes being greater than that following stimulation of the right vagus. In his complete paper Gaskell<sup>7</sup> (19) says of his experiments on *Testudo graeca* that stimulation of the right vagus always brought the heart to a standstill, while stimulation of the left nerve with a current of the same strength was less effectual. Even a strong stimulus applied to the left vagus did

<sup>3</sup> McWilliam (13), *loc. cit.*, p. 217.

<sup>4</sup> McWilliam (13), *loc. cit.*, p. 223.

<sup>5</sup> McWilliam (13), *loc. cit.*, p. 220.

<sup>6</sup> Gaskell (14), *loc. cit.*, p. 1012.

<sup>7</sup> Hofmann (15), *loc. cit.*, p. 160.

<sup>8</sup> Gaskell (19), *loc. cit.*, p. 82.

not affect the rate. Mills (20, 21) showed that stimulation of the right vagus nerve readily stopped the heart in *Emys lutaria*, while stimulation of the left nerve had no influence whatever;<sup>8</sup> in *Testudo graeca* stimulation of each vagus produced identical effects.<sup>9</sup> It will be seen that his results differ slightly from Meyer's and entirely from those of Gaskell. In a later paper Mills (21) reported<sup>10</sup> that effects of stimulation of the two vagi in the Slider terrapin (*Pseudemys rugosa*) were exactly alike in five cases, the right vagus was much more effective over the rhythm in two and slightly more effective in four cases, while the left nerve was without effect on the rhythm in two cases. He concluded that the right vagus controls the rate better than the left, though in some cases the reverse is true. The left nerve sometimes had no effect on the heart, or an effect only on its force. He added an observation<sup>11</sup> which was corroborated later by Garrey in a slightly modified form; i. e., that the vagi have a homolateral but never a crossed distribution. Kazem-Beck (22) in 1888 was able to arrest the heart of *Emys caspica* in seventy-five animals out of seventy-seven.<sup>12</sup> In the remaining two he caused a negative inotropic but no chronotropic effect. On stimulating the left vagus he caused a stoppage of the heart in forty-seven animals, a negative inotropic effect in ten, and no effect in twenty. Kronecker (23), working on *Testudo graeca*, found that "very often only the right nerve was active and stimulation of the left was without effect, just as has been described by Meyer. In many other animals the activity of both nerves was equal."<sup>13</sup> Of another tortoise, *Cistudo europaea*, Guyénot (25) says in his conclusions:<sup>14</sup> (1) Only the right pneumogastric acted on the rate of the heart. It diminished the tone of muscle in diastole, but the force of the systoles was not modified. (2) The left vagus did not act on the rate. It decreased the tone in diastole and perhaps also in systole, the force of the latter being also diminished. (3) Exceptions to these observations were found. Garrey (2) has recently shown that "the right vagus exerts a chronotropic effect, as well as other effects, upon the right veins and produces quiescence of the auricles and ventricles mainly through its chronotropic effect, rarely, with weak stimuli, the effect is brought about by production of a sino-auricular block, although in these cases the inotropic and other effects are also apparent. . . . But the left vagus decreases the excitability, conductivity, and contractility of the auricles to a point at which they fail to respond to the stimulus of the pace-maker, thus no impulses can pass through to the ventricle."<sup>15</sup>

Mills (26) studied the effects of stimulation of the two vagus nerves in several varieties of sea turtle and reported that "a comparison of these results with those reported for the terrapin will show a great resemblance. In by far the larger number of cases the right vagus has greater inhibitory power than the

<sup>8</sup> Mills (20), *loc. cit.*, p. 360.

<sup>9</sup> Mills (20), *loc. cit.*, p. 361.

<sup>10</sup> Mills (21), *loc. cit.*, p. 247.

<sup>11</sup> Mills (21), *loc. cit.*, p. 249.

<sup>12</sup> Kazem-Beck (22), *loc. cit.*, p. 348.

<sup>13</sup> Kronecker (23), *loc. cit.*, p. 215.

<sup>14</sup> Guyénot (25), *loc. cit.*, p. 1033.

<sup>15</sup> Garrey (2), *loc. cit.*, p. 341.

left, exceptions to this, though few, sometimes occurring. In the marine turtle no case in which the left vagus was wholly without effect on the rhythm was found."<sup>16</sup> He added, "I would then explain the greater effect of the right vagus, as a rule, by the character of the contraction wave, associated with the right part of the sinus and its associated veins, and by the fact that the nervous supply to this seems to be chiefly from the right vagus, rather than to any deficiency in the kind and number of the inhibitory fibers in the left vagus; both may supply an equal number of such fibers, but if the supply be even partially unilateral, then the results follow as I have endeavored to explain."<sup>17</sup>

In regard to snakes (*Genus tropidonotus*), Mills (27) came to the following conclusions: "(1) In no case was either vagus without effect on the rhythm of the heart. In every case actual showing, and, with a sufficiently strong current, arrest followed stimulation. (2) In the majority of instances the right vagus was more efficient than the left. (3) In a very few cases both nerves seemed to be almost, if not quite, equally influential over the heart's rate." And he adds that the vagi of the snake resemble those of other cold blooded animals he had examined.

In crocodiles Gaskell (28) found that "both right and left nerves caused an absolute and a long continued standstill even with a weak stimulus."<sup>18</sup>

Great variations in the results are found in the reports of stimulation of the vagi in birds. Jürgens (29) in his experiment on pigeons paid no attention to the differences between the effects of stimulation of the two nerves, but noted on his curves the nerve that had been faradized in each instance, and it is evident that stimulation of the right vagus with currents of moderate strength affected the auricles and occasionally the ventricles, but the latter, if arrested, sometimes escaped. Stimulation of the right vagus with weak currents produced only negative inotropic effects on the ventricle. Where the left vagus was stimulated, inotropic and negative chronotropic effects on both auricles and ventricles were obtained. It appears, therefore, that the ventricles sometimes were affected by stimulation of the right vagus and also, possibly more powerfully, by stimulation of the left vagus. Stübel's (30) tables showing the effects of stimulation of the vagus nerves in a variety of birds throw no light on the subject. For the purpose of the present investigation, the observations are not useful, for in many instances the two nerves were not compared in the same animal.

The action of the two vagus nerves has been studied in a variety of mammals. In the horse Arloing and Tripiér (31) found<sup>19</sup> that stimulation of the left nerve caused a stoppage equal to the duration of three pulsations; the pulse then continued to beat with a little more force than had existed before the stimulation; during the passage of the current fifteen pulsations were counted. Stimulation of the right nerve resulted in stoppage equal to six normal pulsations, and when contractions reappeared they seemed to have great force, but only seven were counted as against fifteen during left vagus stimulation. In

<sup>16</sup> Mills (26), *loc. cit.*, p. 11.

<sup>17</sup> Mills (26), *loc. cit.*, p. 12.

<sup>18</sup> Gaskell (28), *loc. cit.*, p. 22.

<sup>19</sup> Arloing and Tripiér (31), *loc. cit.*, experiment ix, p. 421.

short, stoppage was twice as long when the right nerve was stimulated as when the left was stimulated. The difference between the action of the nerves was not absolutely constant. Sometimes the usual action was reversed, and an example of this is described. Only a quantitative difference between the two nerves was found. Dissections were carried out with the view of studying the distribution of the two nerves to the heart. They found that the right vagus supplied a greater number of fibers, and on account of this preponderance they explained the greater influence that the stimulation of the right vagus has on the heart.

In cats McWilliam (5) found that on stimulation of the inhibitory structures "the ventricles sometimes give only one beat in response to every second or third auricular beat."<sup>20</sup> He showed further that in some cases there was ventricular standstill while the auricles went on beating. "The excitability of the ventricles, as tested by single induction shocks applied to their surface, seemed unimpaired. Moreover, mere weakness of the auricular beats would not account for the non-response of the ventricles; for very much weaker auricular beats than those observed in the above mentioned cases are quite sufficient, in the uninhibited heart, to determine the occurrence of responsive ventricular contractions. The failure of ventricular sequence was in all probability due to an interference with a mechanism of propagation quite different from the pressure changes caused by the auricular beats." Although he observed phenomena like those described in the present study as arising from stimulation of the left vagus, he did not conclude that differences existed between the two vagi, but regarded them as equally potent.

In dogs observations have been made by numerous observers, Knoll (32), Arloing and Tripier (31), Pawlow (33), Roy and Adami (7), Bayliss and Starling (8), Einthoven (34, 35), Hering (10), Dogiel (36), and Rothberger and Winterberg (11, 12).

Knoll (32) states that in three of his animals (one of which was not curarized) "there was no increase in the rate of the heart or in the height of the blood pressure after the vagi were cut." In a footnote he adds, "In one case both appeared clearly after cutting one vagus, while cutting the second contributed no added effect. As Prof. Hering tells me, this happens often in rabbits and dogs, and also stimulation of one vagus at a given strength yields an effect on the heart unequal to that of the other, even if all the conditions necessary have been observed. All this shows that in mammals, too, the inhibitory fibers of the heart are divided unequally between the two vagi."

Arloing and Tripier (31, 37) cut the medulla in a dog and found<sup>21</sup> that stimulation of the left vagus nerve did not stop the heart, while under the same conditions, stimulation of the right vagus led to complete arrest followed by slowing. The right nerve was more sensitive to the action of the current than the left. In two other experiments the peripheral end of the left nerve, the right nerve apparently being intact, was stimulated and the heart stopped for fifteen seconds, but contraction began again and thirty-eight of them were counted during the remaining portion of the period of stimulation. Legros and Onimus (38) attempted to show that the results of stimulating the right and

<sup>20</sup> McWilliam (5), *loc. cit.*, p. 189.

<sup>21</sup> Arloing and Tripier (31), *loc. cit.*, experiment viii, p. 421.

left pneumogastrics were too inconstant to permit the conclusion of Arloing and Tripier that differences exist between the two nerves. They based their conclusions on observations made on rats, guinea pigs, dogs, rabbits, frogs, and tortoises.

Pawlow (33) dealt chiefly with the results of stimulating the large inner branch and other chest branches of the right vagus nerve.

Roy and Adami (7), in their summary of the action of the vagus nerves in the dog, stated<sup>22</sup> that: "(1) With relatively weak excitation,<sup>23</sup> there is slowing of the rhythm but no irregularity. (2) With stronger stimulation the independent ventricular rhythm shows itself, and this, by interference with the ordinary or auricular rhythm, usually leads to irregularity of the ventricles, and, in any case, a want of complete coördination between auricles and ventricles; in other words, there is a physiological irregularity of the heart. (3) With stronger vagus excitations the auricles have either ceased to beat or the impulses which pass from them to the ventricles are too weak to excite the latter to contraction, and the ventricles are left entirely to contract by their independent intrinsic mechanism, and are completely freed from the control of the vagus, save that this nerve has still some control over their excitability." These writers did not distinguish between the effects produced by stimulating the two nerves.

Bayliss and Starling (8) obtained results similar to those of Roy and Adami, and apparently they also regarded both vagus nerves as having identical functions. In their experiments the right nerve apparently remained intact and the left one was cut. They stimulated with strong currents, and "thought" that the auricles had entirely ceased beating and that the ventricles were beating with their own proper rhythm." They consider it "possible that an excitatory process may occur in the auricle and may be transmitted thence to the ventricle without causing any appreciable (i. e., registrable) contraction of the auricle itself." Further on in their paper they made the observation<sup>24</sup> that stimulation of the vagus can cause incomplete heart-block when the ventricles "only respond every now and then to an auricular beat."

Einthoven (34, 35) studied with the string galvanometer the results of stimulating the vagus nerves. In his first paper (34) he has reproduced a curve of right vagus stimulation (figure 33) and another curve (figure 34) which resembles those we have obtained following stimulation of the left nerve, but he does not mention which nerve was stimulated. His later paper (35) added no further data on the differences between the two nerves.

Hering (10) has published curves (figures 5, 6, 7, and 8) showing ventricular silences following vagus stimulation; that is, there was incomplete heart-block, usually at the ratio of 2:1. One of his curves (figure 5) indicates the occurrence of a high degree of block, but this occurred only when the left vagus nerve was stimulated.

Dogiel (36) states that<sup>25</sup> "many physiologists have called attention to the fact that stimulation of the right vagus has not the same effect on the heart

<sup>22</sup> Roy and Adami (7), *loc. cit.*, p. 237.

<sup>23</sup> Of the vagus nerve in the dog.

<sup>24</sup> Bayliss and Starling (8), *loc. cit.*, p. 410.

<sup>25</sup> Bayliss and Starling (8), *loc. cit.*, p. 412.

<sup>26</sup> Dogiel (36), *loc. cit.*, p. 122.

action as stimulation of the left, a result due in part to the strength and duration of the stimulus."

Rothberger and Winterberg (11), in one experiment which they have described in detail, observed<sup>22</sup> that "the effect of a simple stimulation of the two vagi differed in so far that stimulation of the right caused complete stoppage, while stimulation of the left vagus caused only slowing of the auricular contractions with complete cessation of the ventricular (figure 7 b)." Their comment on the result obtained in this experiment is<sup>23</sup> that "exceptionally the inhibitory nerves for the sinus node pass exclusively in the right, for the atrioventricular groove, on the other hand, in the left vagus." And finally they state,<sup>24</sup> "Inhibitory fibers for the left auricle and the atrioventricular groove are usually so divided that inhibitory effects can be obtained by stimulating either vagus. Only in a few cases do only negative chronotropic fibers for the primary pace-maker pass in the right vagus."

François-Franck (6) states<sup>25</sup> that he has seen that "stimulation of the vagus nerve can suppress ventricular while auricular contractions continue. To bring about this result induced currents were used, weak enough not to cause complete arrest of the whole heart. These stimuli, for example, slowed the ventricular beats by five, while they caused the auricles to lose only two systoles in five. Three auricular beats occurred then during a long ventricular pause." The result is like that due to stimulation of the left vagus, which is described in this paper.

Hunt and Harrington (39) were able to produce incomplete heart-block in the calf by stimulation of the vagus nerve, but they fail to mention which one was used.

Before the publication of the work of Mills and Garrey, Masoin (40) in 1872 seems to have been the only experimenter who had insisted that this question of difference between the vagi involved that of the essential asymmetry of the body as far as the vagus nerves are concerned. In his experiments on rabbits he found that stimulation of the right nerve was more effectual in slowing the rate of the heart than stimulation of the left. He states, however, that the two nerves have no special sphere of action and so denied the existence of qualitative differences between them.

The vagus nerves in rabbits have been studied by Langendorff (41), Johansson and Tigerstedt (42), and by Rehfisch (9). Langendorff (41) experimented on sixteen rabbits. In three animals stimulation of each vagus had an equal effect on the heart; in three animals the right vagus was more effectual; in eight, the left; and in two sometimes one and sometimes the other was more effectual. The difference in effect of the two nerves was determined by the degree of slowing produced. Johansson and Tigerstedt (42) working on rabbits confirmed the work of McWilliam (5) on cats. They stimulated only the left vagus nerve and did not register the auricular contractions. They found that weak currents only slowed the ventricles (and the auricles), but that currents

<sup>22</sup> Rothberger and Winterberg (11), *loc. cit.*, p. 356.

<sup>23</sup> Rothberger and Winterberg (11), *loc. cit.*, p. 361.

<sup>24</sup> Rothberger and Winterberg (11), *loc. cit.*, p. 372.

<sup>25</sup> François-Franck (6), *loc. cit.*, p. 403.



of increasing strength not only slowed but decreased the strength of ventricular contractions until they ceased to beat. Rehfsch (9) found<sup>21</sup> that "a fifth effect of vagus stimulation\* is a single or succession of ventricular silences, while the auricles continue to beat (see figure 5)." Two explanations were offered for this phenomenon. The first explanation, that it was the result of negative dromotropic effects on the A-V bundle, was rejected because there was no delay in the As-Vs interval, either in the cycles which preceded or in the cycles which followed the ventricular silence. He favored a second explanation; namely, that there was either a diminished strength in the conduction impulse or a diminished irritability in the ventricular or the A-V systems.

In guinea pigs Harrington (43) found<sup>22</sup> both vagi to be always efficient and in no case was he able to discover any greater efficiency of one than of the other nerve.

The action of the two vagus nerves in the human subject has also been investigated. The method of investigation has consisted in exercising digital pressure along the course of the intact nerve in the neck. This method was described by Czermak in 1865 and its value has been demonstrated repeatedly since then. This method was employed by Robinson and Draper (3) in a comparison of the action of the two nerves in man, and they found that differences similar to those about to be described in this paper could be elicited. It must be stated that the method is uncertain, and uniform results cannot always be expected.

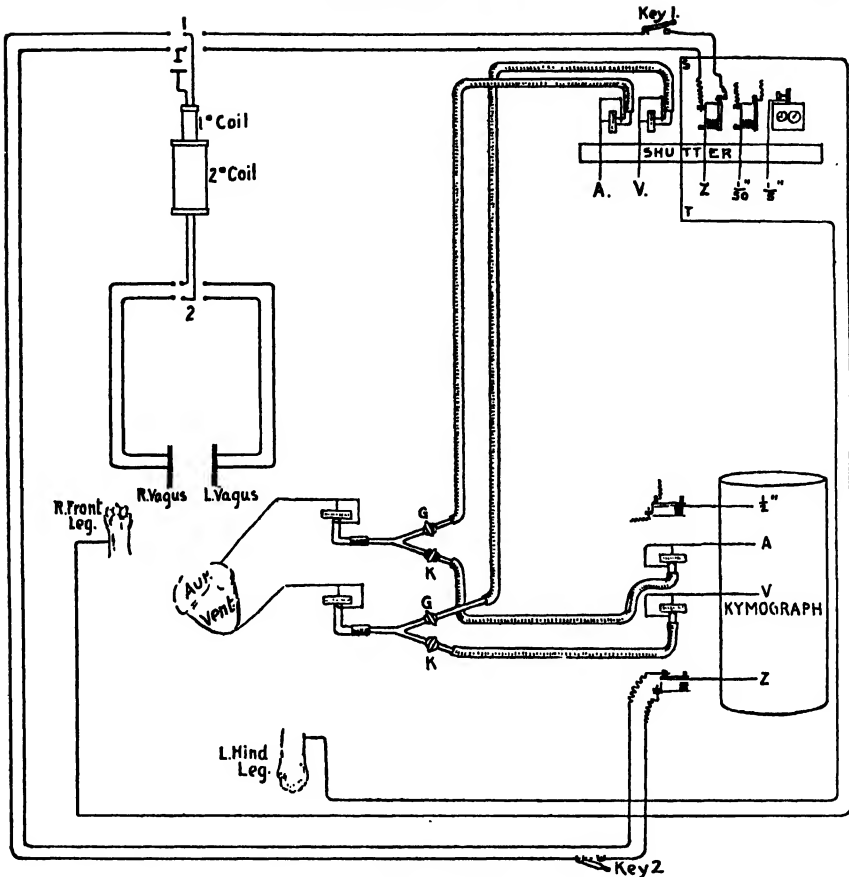
#### METHODS.

In this investigation only dogs of medium size were used, weighing usually from 5 to 7 kilos; a few weighed from 10 to 12 kilos. The dogs were first anesthetized with ether in the ordinary way; no other drugs were used. When complete anesthesia had been established, a catheter was introduced through the mouth, larynx, and trachea, after the manner described by Meltzer and Auer, and through this air and ether, in regulated quantities, were administered. The pressure in the tube just before it entered the mouth was read on a mercury manometer which was connected with the catheter by a side arm. A pressure of about 20 mm. of mercury was maintained. When the chest was opened, if the animal was well anesthetized, the lungs appeared in a semicollapsed condition, but they evidently contained sufficient air, for even after operations lasting four to five hours the animals were in excellent condition. The fact that the lungs were collapsed and practically motionless facilitated the use of a suspension apparatus to register the motion of both auricles and ventricles. This was accomplished in the usual way by means of hooks from the right auricle and right or left ventricle, or by means of Roy and Adami myocardiographs leading to two receiving tambours, which were connected with writing tambours by air transmission. The writing tambours were four in number, two arranged to write on a Reickert kymograph and two hung before the shutter of a photograph box. One pair or the other could be set in motion by opening and closing stop-cocks in two Y tubes (text-figure 1), placed, one each, in the transmitting tubes

<sup>21</sup> Rehfsch (9), *loc. cit.*, p. 170.

\* Single induction shocks were used.

<sup>22</sup> Harrington (43), *loc. cit.*, p. 393.



TEXT-FIG. 1. The connection of the dog with the recording apparatus is shown. The auricles, ventricles, two vagus nerves, right fore, and left hind leg are indicated. Registration on the kymograph is effected when the stop-cocks G are closed and the stop-cocks K are opened, and when the double throw-switch 1 is thrown to the right. Right and left vagi are stimulated according to whether double throw-switch 2 is thrown to right or left. Registration is effected on sensitive paper behind the "shutter" of the photograph box, when stop-cocks K are shut and stop-cocks G are opened and when the double throw-switch 1 is thrown to the left. Keys 1 and 2 complete the circuit by which stimulation of the nerves is effected. A signal, Z, indicates the duration of the stimulus. A and V represent the tambours registering the motions of the auricle and ventricle, one each before the shutter and before the kymograph. A time-marker recording one half seconds was employed for the kymograph; two time-markers recording 0.02 of a second interruptions made by a tuning fork and 0.2 of a second made by a Jacquet clock were employed for the photographic records. The right fore and the left hind leg are shown connected with the string, ST, of the galvanometer.

from auricles and ventricles. On the kymograph, besides the motions of auricles and ventricles, was written the time in one half seconds and the record of faradic stimulation by a signal in connection with the primary coil of a Harvard inductorium. The primary coil was supplied by a dry cell, known always to have a potential of two volts and an amperage not below twelve. In a number of experiments in the beginning the suspension methods alone were used. Later curves were made with a string galvanometer (Edelmann's larger model). By means of the device already mentioned, the tambours hung before the shutter of the photograph box could be set in motion, and the shadows thrown by their writing points were photographed on sensitive paper together with the oscillations of the galvanometer string. In many experiments only galvanometric records were obtained. Three kinds of electrodes were used to connect the dogs with the galvanometer. At first those devised by Gotch for taking direct leads from the heart were employed. Although electrodes of this form may attain much flexibility and usually follow the motions of the heart well, they were soon discarded for two reasons. First, the rapid motions of the hearts of dogs make it very difficult for the worsted threads to maintain satisfactory contacts with the surface of the uninjured heart, and owing to these motions distortions of the curves frequently occurred and sometimes interfered with their interpretation. In the second place, the curves thus obtained differed so much, both in size and shape, from those with which we are familiar that their interpretation was difficult. The second form of electrode used was that recommended by Einthoven for dogs. The legs were wrapped with gauze saturated with salt solution, the end of the gauze lying in a vessel of salt solution. In this solution a porous vessel containing zinc sulphate solution was placed. From a zinc plate in the latter solution, copper wires led to the galvanometer. The third form of electrode used was the simplest. It consisted of plates of German silver wrapped on both sides with felt or cotton saturated with salt solution. These were placed around the legs of the animal. Binding posts soldered into the German silver plate formed the point of connection between the electrodes and the galvanometer. The second lead (right fore leg and left hind leg) was used in all these experiments. Curves have been obtained with the chest unopened and also opened, and by direct and indirect leads. It may be stated that for the purposes of this investigation the results did not differ with the different methods.

The greatest amount of information was obtained when the galvanometric and the suspension methods were combined. The diminution in the muscular activity of the heart which takes place during stimulation of the vagus nerves is frequently so great that an inspection of suspension curves or of curves of muscular shortening leads to the belief that all cardiac activity has ceased. During such periods, however, it is quite apparent that cardiac activity may, and in fact does often continue, and causes distinct and sometimes large oscillations of the string of the galvanometer. Without electrical tracings certain of the experiments of this series would not have yielded the results which are now presented, and some of the general rules deduced could not otherwise be stated. On account of the relatively slight value, therefore, of suspension curves and on account of the complications due to an extensive operation on the chest, the results of a number of the experiments are based on galvanometric curves only.

The experiments in which only suspension curves were obtained or in which the galvanometric curves were unsatisfactory have been discarded.

The curves made by the oscillations of the galvanometer string were photographed directly on bromide paper. Above the curves showing the movements of the galvanometer string there were photographed the movements of the tambour levers recording auricular and ventricular contractions, and below it, first a signal indicating the period of stimulation of the vagus nerve, then the rapid motions of a tuning fork, a double motion of which equals 0.02 of a second, and finally a curve the peaks of which show the movements of the lever of a Jacquet clock 0.2 of a second apart. Whenever possible, both methods of marking time were employed to facilitate subsequent measuring. The photographed curves then show from above downward (1) the curves of the auricular and (2) of ventricular contraction, (3) the curve of the galvanometer string, (4) the stimulation signal, (5) a time curve in 0.02 of a second, and (6) a time curve in 0.2 of a second.

In every experiment both vagosympathetic nerves were dissected without injury, as far as this was possible, and each was laid and secured in a shield electrode. To prevent a spread of the stimulating current the nerves and shield electrodes were surrounded by a rubber membrane which was kept dry by laying it on a pad of gauze placed in the groove occupied by the carotid sheath. The muscles and skin were then drawn over the nerves. In most instances spread of the current was satisfactorily prevented and the nerves remained in functional activity throughout the period of the operation.

Curves were always taken either on the kymograph or photographically before opening the chest and also when the chest was opened and before section of the vagus nerves. Sometimes curves were taken before and after the section of each vagus nerve. A number of curves were usually taken showing the effects on the heart of faradic stimulation of each vagus nerve. The curves were so taken that they included the heart action before stimulation, as well as that during the entire period of stimulation, and they were continued until the heart returned to a condition approximately the same as that before stimulation. The first portion of each curve serves as a control.

The strength of the stimulating current was usually the same. One two volt cell fed the primary coil of the inductorium and the secondary coil was in almost all the experiments placed fifty millimeters from the primary coil. Exceptions to this rule are noted where they occurred. In a given case the conditions of stimulation throughout the experiment were kept constant, so that inequalities from this source cannot be held responsible for the differences in effect which have been observed. It is conceivable that, were the strengths of the current used on the two sides varied, identical results on stimulation might have been obtained. It is believed, however, that enough evidence is at hand to show that such would not be the case. A report bearing on this point will be made later.

## RESULTS.

In the course of this investigation fifty-four experiments were performed. Differences in the action of the two nerves were found in forty-eight (88 per cent.) of the dogs. Stimulation of the right vagus nerve arrested all portions of the heart in fifty-one experiments. These observations do not differ from

trates this effect. The stimulus lasted for 6.5 seconds. The whole heart ceased to beat during the period of stimulation and for about 1.2 seconds longer. The latent period was 0.4 of a second, about equal to the duration of a cardiac cycle. The As-Vs (P-R) interval before stimulation equalled 0.09 of a second, and for the first four cycles after stimulation lengthened to 0.1, 0.11, 0.12 and 0.11 of a second. The P wave was small at first, but later became diphasic. This phenomenon has been described by Einthoven.

Numerous differences in detail from the curve (figure 1) which has been taken as characteristic of the result of right vagus stimulation were noticed. Occasionally the P-R interval remained unchanged after the cessation of the stimulus, while in some experiments it lengthened as much as 0.05 of a second. In other instances, an auricular contraction, that is to say a P wave, not followed by a ventricular contraction, occurred after the stimulus began to be effectual. An instance of this is shown in figure 2, experiment 666. In the same curve two complete cycles appear during stimulation of the right vagus nerve, and between the two a blocked auricular contraction, P', is seen. The appearance of a blocked auricular systole in this situation during stimulation was relatively rare. The P-R intervals lengthened during the period of stimulation from 0.1 to 0.12 and 0.14, but afterward they fell again to 0.11 and 0.1 of a second. A blocked auricular systole, P', is shown in figure 3, experiment 666, but here it occurs after the cessation of stimulation following the first returning cardiac cycle. A blocked auricular systole in this situation was not infrequent. A slight increase (0.1 to 0.12) in the P-R time occurs in this curve. After stimulation the R waves increased in size.

It has been mentioned that during stimulation of the right vagus nerve, the ventricles sometimes continued to contract, although the auricles had ceased. When this occurred, the origin of the ventricular contractions was ectopic. These escaped ventricular contractions differ, therefore, from the ventricular contraction seen in figure 2. Sometimes only a few ventricular contractions appeared, but occasionally a relatively rapid idioventricular rhythm arose. In figure 4, for instance, experiment 659, only two ectopic ventricular contractions escaped at irregular intervals during a period of stimulation lasting 6.5 seconds. The complexes correspond to contractions originating in the right ventricle. This curve shows again the occurrence of a blocked P' wave after the beginning of effective stimulation, and also blocked P' waves after the end of stimulation. The increase in the P-R time in the first returning cycle increased more than was usual, 0.1 to 0.18 of a second. In figure 5, experiment 661, ectopic ventricular complexes appear, the result in this instance of contractions arising in the wall of the left ventricle. These occur at fairly regular intervals and develop a rapid rate, sixty per minute. No evidence of auricular contraction can be seen during the period of stimulation, but at its cessation, three P waves appear before a nomotopic ventricular complex occurs. The P-R time is slightly increased, 0.11 to 0.14 of a second. The P waves have a tendency to be diphasic. It is not quite clear why the first returning P wave fails to be followed by a nomotopic R wave. The explanation probably is to be found in the fact that conductivity was much depressed and the impulse reached the ventricular muscle after the ectopic pace-maker had discharged its stimulus, and P' found the ventricle refractory. In figure 6, experiment 639, precisely similar events are

shown as in figure 5, the difference between the two consisting in the fact that the ventricular complexes originated in the right ventricle and beat at a rate of 92.4 per minute.

An effect which resulted from stimulating the right vagus and which was seen only once in this series of experiments (experiment 625) consisted in an unusual slowing of both auricles and ventricles. They were slowed not in the usual way in which the sequence of contraction is maintained, but for three cycles during the period of stimulation, the P waves fell between the R and T waves. The R-P interval was too short to permit the interpretation that a reversal (Vs-As) of the normal rhythm had probably occurred; the unusual sequences probably resulted rather from the fact that both auricles and ventricles received their impulses from the junctional tissues lying between them and the course of the stimulus flowed upward and downward to each. Direct leads were used in this experiment so that deductions are not drawn from the form of complexes found, although the form of the P wave was altered during the period of stimulation.

The simplest effects on stimulating the left vagus nerve are seen in figure 7, experiment 662. During a period of stimulation lasting 7.8 seconds there occurred, first, a reduction in rate from 135 to 114.2 beats per minute, and then, an increase in the time of conduction from auricle to ventricle (P-R interval) from 0.1 to 0.24 of a second. The P-R time lengthened in general from the beginning to the end of stimulation. Toward the end of stimulation, the P waves tended to become diphasic, an effect noticed also on stimulating the right vagus nerve. A small positive wave before and immediately connected with the P wave also appeared during the time of stimulation. The split P wave has been noticed<sup>10</sup> by Hering (10). In view of the general dissociative effects described in this study as resulting from stimulation of the left vagus nerve, some degree of contributory evidence is lent to Hering's surmise that this wave represents a succession in the contraction of the right and left auricles. It is to be noticed that the effect of the stimulus lasted more than 3.5 seconds after its cessation.

A more complicated effect which results from left vagus stimulation is illustrated in figure 8, A and B, experiment 666. The rate of the whole heart was reduced from 173.6 to 113.2. The slowing was progressive, as can be demonstrated by the increased time between the auricular contractions, the P-P waves. Before the application of the stimulus, this interval was 0.35 of a second, but it gradually lengthened to 0.55 of a second. About two seconds after the end of stimulation the P-P interval was normal again. A further effect consists in the occurrence of blocked auricular contractions (P') during the period of stimulation. In figure 8, A and B, six blocked P' waves are seen. The P-R interval in this curve, as in digitalis poisoning, gradually increased until an auricular wave (P') failed to be followed by an R wave. Progressive increase in P-R time of this nature occurred in the first three groups of cardiac cycles, but in the fourth the progression was not uniform (0.11, 0.15, 0.14, 0.17, and 0.14 of a second). As the length of the stimulation increased, all the P waves became diphasic.

Finally the most marked effect that is seen as the result of stimulating the left vagus nerve is illustrated in figure 9, experiment 673. During a period of stimulation lasting 7.2 seconds, the auricular contractions continued uninter-

<sup>10</sup> Hering (10), *loc. cit.*, p. 158.

ruptedly, but the rate was reduced from 176.64 to 79.56 per minute. The ventricular contractions, however, ceased for nine seconds; that is to say, throughout the period of stimulation and for some time after it. The first ventricular contraction to return after the end of the stimulus did so two seconds after stimulation had ended and 0.12 of a second after the preceding P wave. The P' which follows the ventricular beat was blocked, but the sequential contractions of the heart were then resumed. The P-R interval became longer, 0.14 of a second, than it had been before stimulation, but it gradually returned to normal. The R waves increased in size from six to eight millimeters for six cycles and then suddenly returned to the original height. The curve also shows that during stimulation seven or possibly eight waves can be identified in the suspension curve of the right auricle. These become progressively smaller and finally disappear. The corresponding P waves in the electrocardiogram, on the contrary, become a little larger near the end of stimulation and can, in fact, be identified without difficulty. It is this gradual disappearance of the waves of the suspension curve, while the electrical ones remain, which made it necessary to discard those experiments in which only the former were made.

Another example of this degree of effect of left vagus stimulation is seen in figure 10, A and B, experiment 667. Except for the preliminary arrest of the auricular (2.3 seconds), as well as of the ventricular contractions, the results of stimulating the left vagus were similar to those seen in figure 9. The auricular rate was about 96. After the cessation of stimulation, its effect continued in a form previously described. At first one ventricular contraction took place long (0.48 of a second) after the auricular beat preceding it. This ventricular beat was probably ectopic, especially since its size is greater than that of the other complexes. A ventricular beat did not appear again until five more auricular contractions had occurred. A succession of sixteen ventricular contractions followed this one, each preceded by two auricular beats, producing incomplete heart-block at a ratio of 2:1. One sequential cycle and another ventricular contraction following two auricular systoles followed, when the normal rhythm was finally resumed. The P-R interval was 0.12 of a second before stimulation; it reached 0.2 of a second during the period of incomplete block, and twenty seconds after stimulation it was still 0.16 of a second. So great an after effect, lasting so long a period, was observed only in this experiment.

In the experiments just described, complete ventricular asystole was present throughout the period of stimulation. But in a number of experiments, ventricular quiescence did not take place. Normal ventricular complexes disappeared and one must conclude that normal ventricular contractions had ceased. The complexes representing the ventricular beats which continued have the form of those arising in the wall of the right or the left ventricles, and are assumed to be ectopic in origin. They were the result of an idioventricular rhythm arising because impulses failed to reach the ventricles on account of the depression in conductivity of the auriculoventricular system during stimulation of the left vagus nerve. In so far as the ventricles under these circumstances did not receive supraventricular impulses, they are comparable to experiments on auriculoventricular block.\* Figure 11, experiment 639, illustrates this reaction

\* The relation of the two vagus nerves to heart-block after it has been established is not discussed in the present communication.

to stimulation of the left vagus nerve. The stimulus lasted 6.3 seconds. During this time the auricular beats continued at a rate of about 127. The usual ventricular complexes changed to a new form, which was maintained during stimulation and for one second afterwards. One second was about the average length of time that the effect continued after stimulation had ceased. The rate of the ectopic ventricular beats was about 86. The curve shows that when the ventricular contractions were ectopic, they occurred in complete dissociation from those of the auricles. Suspension curves alone would only have disclosed the fact that dissociation between auricular and ventricular contractions had taken place. But the electrical curves make it clear that dissociation in the usual sense did not occur, for in that event the form of the ventricular complexes would not have been altered. An explanation of why they are changed is difficult and the hypothesis which follows is offered tentatively. In cases of clinical heart-block the anatomical lesion, which is the cause of the block, usually involves only a short portion of the main stem of the conduction bundle; the right and left branches are normal, and are left in continuity with the ventricular muscle. The ventricular muscle continues to receive impulses to contraction propagated to it apparently over the usual course provided by the two branches. But there are cases, both clinical and experimental, published by Eppinger and Rothberger (44), Eppinger and Stoerck (45), and by Cohn and Lewis," in which lesions of the branches of the bundle rather than in the main stem have been demonstrated. In such cases, propagation of the impulse over the normal pathway is obviously impossible and some abnormality in stimulus production and stimulus distribution must occur. Electrocardiograms taken from these cases have shown the ventricular complexes to be characteristic of contractions arising in the right or left ventricle. It is with these clinical cases that experiments like those illustrated in figure 11 must be compared. If it is assumed that inhibitory fibers, derived from the vagus nerves, are distributed to the auriculoventricular bundle, they are probably distributed to its branches. The work of Engel (46), following that of Wilson (47), showing that nerve fibers are present in the branches makes this assumption more probable. Then if stimulation of the left vagus nerve produces an effect on the main stem of the bundle, it follows that the effect is probably continued downward along its branches. If this view of the distribution and function of the vagus nerves is correct, stimulation of the left nerve may depress the conductivity of the muscle of the main stem and branches of the A-V system to a degree so great that the ventricular muscle will receive no impulses conveyed by them. The ventricular muscle is then reduced to a condition directly comparable with that in those cases in which structural lesions in the branches of the A-V system have been demonstrated and in which the ventricular muscle was obliged to originate stimuli, if it originate them at all, in an atypical fashion and to distribute the stimuli over unusual paths yielding electrical curves of atypical form. It is probable that the mechanism suggested explains the occurrence of the atypical form of the curves in this group of experiments.

Kahn (48) had previously demonstrated the fact<sup>28</sup> "that automatic ventricular

<sup>27</sup> This case is to be published in a forthcoming number of *Heart*. References to electrocardiograms of this patient can be found in Lewis, Thomas, *Mechanism of the Heart-Beat*, London, 1911, p. 237.

<sup>28</sup> Kahn (48), *loc. cit.*, p. 633.



contractions which develop during artificial stimulation of the vagus are accompanied by atypical electrical curves." He states that "auriculoventricular dissociation in man, or section of the A-V bundle or its branches in the dog have shown in the investigations which have been made up to the present time that automatic ventricular contractions are expressed in the form of typical electrocardiograms. In dogs, on the contrary, dissociation caused by stimulation of the vagus nerve is accompanied by atypical electrocardiograms, while in cases of poisoning, both atypical and typical forms are found." He did not realize, however, that the phenomenon he was describing occurred only with stimulation of the left vagus. Kraus and Nicolai (49) and also Rothberger and Winterberg (11) have reported observations like those of Kahn. The latter have explained the ectopic ventricular contractions during vagus stimulation as due to a mechanical cause, such as the filling of the ventricular cavities.

It has been stated that when stimulation of the left vagus nerve causes ventricular asystole, presumably by depressing the power of the A-V system to conduct impulses, an independent rhythm will be initiated, depending upon the degree of irritability of some portion of the ventricular muscle. The degree of irritability may be so great that a ventricular rhythm is established practically immediately or within a very short time. On the other hand, the degree of irritability may be so low that the development of an idioventricular rhythm may be delayed until the stoppage has continued for some time. The occurrence of stoppage with the gradual development of an idioventricular rhythm has been studied in connection with the subject of experimental heart-block. Figures 10, A and B, 11, and 12 show degrees of irritability of the ventricular muscle measured by the length of time required to establish an independent ventricular rhythm. In figure 10, A and B, an independent ventricular contraction did not occur until 8.3 seconds after the beginning; that is to say, 0.6 of a second after the cessation of stimulation. None followed after that for about 2.3 seconds, the second one being in response to an auricular contraction, the depression of conductivity in the meanwhile having been relieved. In figure 11 there was practically no stoppage. In figure 12, experiment 644, an intermediate condition is seen. Stoppage of ventricular contractions lasted 2.4 seconds and the second beat followed the first after an interval of three seconds. These were both ectopic beats and they arose in the wall of the right ventricle. Ectopic beats may also arise in the left as well as in the right ventricle; in another curve taken during this experiment there were, in fact, three idioventricular contractions which arose in the left ventricle.

A combination of the effects described was sometimes seen, as in experiment 661 (figure 13, A and B). Stimulation of the left vagus arrested the contractions of both auricles and ventricles. Independent ventricular contractions were established before the auricular muscle escaped from the first effects of stimulation. The efficiency of the stimulus rapidly decreased and sequential contractions occurred, at a reduced rate but with a prolonged P-R (0.1 to 0.14-0.17) interval. In this dog, two varieties of effect due to stimulating the left vagus nerve were obtained. In other dogs, successive stimulations of this nerve resulted in effects first of one kind and later of another. In experiment 666, the first stimulation of the left vagus produced merely an increase in the length of the P-R interval. A second stimulation caused the heart to beat in a two to one rhythm, while a third

stimulation produced quiescence of the ventricular contractions, during which the auricular muscle continued to contract. Between the first and second stimulations nine seconds elapsed and between the second and third, six seconds. Seven other experiments (table I) occurred in this series in which more than one of the varieties of effects resulting from left vagus stimulation were obtained. In these cases the maximum effect was not always obtained the first time the nerve was stimulated.

In two experiments (Nos. 633 and 649) the reverse of the effects usually obtained were seen. In experiment 633, right vagus stimulation caused nomotopic ventricular contractions to cease, while the auricles continued to beat; a few ectopic ventricular contractions occurred. The left vagus in this case stopped the whole heart. In experiment 649, stimulation of the right vagus nerve produced a two to one block, while left vagus stimulation produced the effect usually brought about by stimulating the right nerve.

A single instance, experiment 662, was found in which stimulation of the left vagus nerve produced long periods of rest between groups of cardiac cycles, between successive single cycles, and between a single cycle and a group of cycles (figure 14, A and B). Each period of rest occupied a length of time that was approximately a multiple of the time occupied by a single cycle. The average duration of the single cycles during stimulation was 0.5 to 0.54 of a second; the periods of rest occupied 1.96, 1.6, 1.05, 1.6, and 1.57 seconds, each approximately four, three, or two times as long as the duration of the single cycles. Later in the curve the single cycles occupied 0.57 to 0.68 of a second, and the periods of rest, 0.99 to 1.67; these were again approximately multiples of the former. The fact that the periods of rest appear as multiples of the single cycles renders the interpretation probable that an incomplete block took place between the pace-maker and the rest of the heart. This experiment affords an example of sino-auricular block in the dog's heart. There was also lengthening of the P-R interval (0.1 to 0.17) during stimulation, the longest interval occurring in the first cycles of each group. The single cycles and the first cycles in each group are represented by complexes altered not only in size but in a minor degree also in form. Sino-auricular block has been obtained by Garrey (2)<sup>29</sup> by stimulating the vagus nerve in the turtle's heart, but in his case the phenomenon is described as resulting from the stimulation of the right vagus nerve by weak currents. He considered the block as a chronotropic effect, although he says inotropic and other effects were also present. In the present experiment the effect was obtained with the left vagus nerve, although four seconds after cessation of stimulation of the right vagus, a single cycle dropped out, and a quiescence equal to about the length of two took its place. There was no lengthening of the conduction time in the cycle after the pause.

#### DISCUSSION.

The description of the curves leads naturally to the conclusion that in dogs an essential difference exists between the right and left vagus nerves in their action on the heart. The difference is not merely a difference in degree, it is a more fundamental differ-

<sup>29</sup> Garrey (2), *loc. cit.*, p. 342.

TABLE I.

Experiment No.	Left vagus. <sup>40</sup>						Right vagus. <sup>41</sup>						Experiment No.	Left vagus. <sup>40</sup>						Right vagus. <sup>41</sup>																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																															
	1	2	3		4	o	Inv.	1	2	3	4	o		Inv.	1	2	3	4																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
			A	B															A	B																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																															
618																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																			

<sup>40</sup> Left vagus. Column 1. Experiments in which P-R time was lengthened. 2. Experiments in which incomplete block occurred. 3. A. Experiments in which ventricular asystole occurred. 3. B. Experiments in which ectopic ventricular systoles occurred. 4. Experiments in which both auricular and ventricular beats were slowed. 0. Experiments in which both nerves produced similar effects. Inv. = experiments in which inverted effects were produced by the two nerves.

<sup>41</sup> Right vagus. Column 1. Experiments in which the whole heart stopped. 2. Experiments in which the whole heart first stopped, but later beat at a slowed rate. 3. Experiments in which the whole heart was slowed. 4. Experiments in which the auricular beats were stopped and ectopic ventricular beats continued.

<sup>a</sup> After cessation of stimulation.

<sup>a</sup> During the period of stimulation.

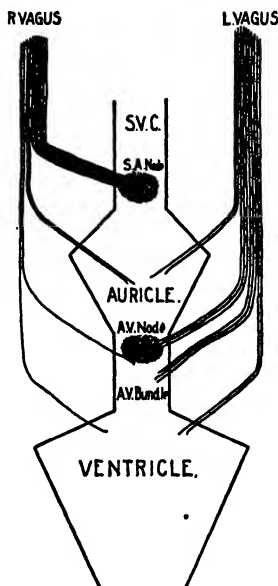
ence. Except in the two cases in which inversion of the usual result was obtained, auricular contractions were always arrested by successful stimulation of the right nerve while stimulation of the left nerve did not stop them. Nor did it ever happen on stimulating the right nerve, even when the rate was only slowed, that the depression of conduction from auricles was so great as to result in the incomplete or complete block of impulses derived from auricular contractions. On the other hand, such phenomena invariably resulted from left vagus stimulation. Other experimenters, already mentioned, have described auricular slowing with ventricular quiescence during vagus stimulation. But as far as the writer is aware it has not been recognized that this combination of phenomena is usually produced by stimulation of the left vagus and not by stimulation of the right. As recently as 1911, Dogiel (36) stated<sup>44</sup> that "many physiologists have called attention to the fact that stimulation of the right vagus has not the same effect on the heart action as the left, a result due in part to the strength and duration of the stimulus"; and von Tabora (50), working in Hering's laboratory on dogs, said that "vagus action alone may possibly on occasions produce ventricular silences, but cannot bring about dissociation."

It is probable that the difference in function between the two vagi is dependent upon the development of the heart and its conducting mechanism, and of the nerves in relation to the muscular structures. An explanation of the differences must therefore await a further development of knowledge regarding these embryological points. The existence of a homolateral distribution of the two vagus nerves in the hearts of turtles has been indicated by Mills (21) and has been proved by Garrey (2). A similar roughly homolateral distribution of the vagi in dogs has also been described by Lim Boon Keng (51), and of the accelerator nerves by von Schumacher (52), but it is doubtful whether the distribution that has been described is as refined as is necessary to explain the results obtained in the experiments described in this paper.

In text-figure 2 an attempt has been made to indicate a possible distribution of the two nerves which might explain their differences

<sup>44</sup> Dogiel (36), *loc. cit.*, p. 122.

in function. Such a distribution, of course, is purely hypothetical and the diagram is offered simply for the purpose of rendering the experimental findings more comprehensible.



TEXT-FIG. 2. The superior vena cava (S.V.C.), the sino-auricular node, the mass of both auricles, the auriculoventricular node and bundle, and the mass of both ventricles are shown diagrammatically. The right vagus nerve is shown to send a preponderating number of fibers to the S.A. node, a smaller number to the auricles and to the A-V system and the ventricles. The distribution on the left side is reversed; none are sent to the S.A. node, and a larger number to the A-V node and A-V bundle and the ventricles.

To each vagus nerve are accredited an equal number of fibers. The greater number of those of the right nerve are represented as entering the sino-auricular node; they exercise, therefore, a preponderating chronotropic effect on the whole heart. A smaller number are distributed to the auricles and to the ventricles, where they exercise inotropic effects far greater on the auricle than on the ventricle. Of the fibers assigned to the left vagus nerve, none enter the sino-auricular node. It might have been more in consonance with the findings in these experiments, however, to have shown a fiber entering the sinus node. Fibers are shown to enter

the auricles where they have an inotropic effect, while others enter the ventricles where they have an inotropic and possibly a bathmotropic action. Most of the fibers are shown to enter the auriculo-ventricular system, for the production of a dromotropic effect is the most conspicuous function of this nerve. It must be added that the diagram, as an explanation even of this paper, does not elucidate all the phenomena found, and is included with hesitancy, lest it be supposed to contain a definitive opinion.

#### CONCLUSIONS.

It may be concluded from the results obtained in these experiments:

1. That stimulation of the right vagus nerve in the dog usually causes arrest of all the chambers of the heart.

2. That stimulation of the left vagus nerve exerts a moderate negative chronotropic effect on the auricles.

3. That stimulation of the left vagus nerve has a profound effect on the conduction of impulses over the auriculoventricular system.

4. That the degree of effect exercised on the auriculoventricular system by stimulation of the left vagus nerve varies. In some dogs conduction is depressed to an extent which causes only a delay in the conduction of impulses from auricles to ventricles (P-R time); in other dogs the conduction is depressed to a degree which results in incomplete heart-block; while in still other dogs conduction is so depressed that although the auricles continue to contract, no impulses pass from them to the ventricles.

5. That when stimulation of either the right or left vagus nerve causes asystole of nomotopic ventricular contractions, ectopic ventricular contractions may occur.

6. That the time which elapses before ectopic ventricular contractions occur depends upon the irritability of the ventricular muscle, and this may vary in different dogs.

7. That stimulation of the left vagus nerve may rarely cause sino-auricular block. Possibly stimulation of the right nerve may also produce this effect.

8. That there is consequently usually a great qualitative difference in the action of the two vagus nerves on the heart of the dog.

## BIBLIOGRAPHY.

1. Meyer, A. B., *Das Hemmungsnervensystem des Herzens*, Berlin, 1869, 1.
2. Garrey, W. E., *Am. Jour. Physiol.*, 1911, xxviii, 330.
3. Robinson, G. C., and Draper, G., *Jour. Exper. Med.*, 1911, xiv, 217; 1912, xv, 14.
4. Cohn, A. E., *Jour. Exper. Med.*, 1912, xv, 49.
5. McWilliam, J. A., *Jour. Physiol.*, 1888, ix, 167.
6. François-Franck, *Arch. de physiol. norm. et path.*, 1890, Series 5, ii, 395.
7. Roy, C. S., and Adami, J. G., *Phil. Tr. Roy. Soc. London*, 1893, clxxxiii, 199.
8. Bayliss, W. M., and Starling, E. H., *Jour. Physiol.*, 1892, xiii, 407.
9. Rehfish, E., *Arch. f. Physiol.*, 1906, Suppl., 152.
10. Hering, H. E., *Arch. f. d. ges. Physiol.*, 1909, cxxvii, 155.
11. Rothberger, C. J., and Winterberg, H., *Arch. f. d. ges. Physiol.*, 1911, cxli, 343.
12. Rothberger, C. J., and Winterberg, H., *Arch. f. d. ges. Physiol.*, 1910, cxxxv, 506.
13. McWilliam, J. A., *Jour. Physiol.*, 1885, vi, 192.
14. Gaskell, W. H., *Phil. Tr. Roy. Soc. London*, 1883, clxxxiii, 993.
15. Hofmann, F., *Arch. f. d. ges. Physiol.*, 1895, ix, 139.
16. Guyénot, E., *Compt. rend. Soc. de biol.*, 1907, i, 1145.
17. McWilliam, J. A., *Jour. Physiol.*, 1885, vi, p. xvi.
18. Gaskell, W. H., *Jour. Physiol.*, 1882, iii, 369.
19. Gaskell, W. H., *Jour. Physiol.*, 1883, iv, 43.
20. Mills, T. W., *Jour. Physiol.*, 1884, v, 359.
21. Mills, T. W., *Jour. Physiol.*, 1885, vi, 246.
22. Kazem-Beck, *Arch. f. Anat. u. Physiol.*, 1888, 325.
23. Kronecker, H., *Arch. internat. de physiol.*, 1904-5, ii, 211.
24. Guyénot, E., *Compt. rend. Soc. de biol.*, 1907, i, 1025.
25. Guyénot, E., *Compt. rend. Soc. de biol.*, 1907, i, 1032.
26. Mills, T. W., *Jour. Anat. and Physiol.*, 1886-7, xxi, 1.
27. Mills, T. W., *Jour. Anat. and Physiol.*, 1887-8, xxii, 1.
28. Gaskell, W. H., *Jour. Physiol.*, 1885, vii, 1.
29. Jürgens, H., *Arch. f. d. ges. Physiol.*, 1909, cxxix, 506.
30. Stübel, H., *Arch. f. d. ges. Physiol.*, 1910, cxxxv, 249.
31. Arloing, S., and Tripiér, L., *Arch. de physiol. norm. et path.*, 1871, iv, 411, 588.
32. Knoll, P., *Sitzungsb. d. k. Akad. d. Wissensch., Math.-Naturwiss. Kl., Abt. III*, 1872, lxvi, 195.
33. Pawlow, J. P., *Arch. f. Physiol.*, 1887, 498.
34. Einthoven, W., *Arch. internat. de physiol.*, 1906-7, iv, 132.
35. Einthoven, W., *Arch. f. d. ges. Physiol.*, 1908, cxxii, 517.
36. Dogiel, J., *Arch. f. d. ges. Physiol.*, 1911, cxlii, 109.
37. Arloing, S., and Tripiér, L., *Arch. de physiol. norm. et path.*, 1873, v, 157.
38. Legros, C., and Onimus, *Jour. de l'anat. et de la physiol.*, 1872, viii, 561.
39. Hunt, R., and Harrington, D. W., *Jour. Exper. Med.*, 1897, ii, 723.
40. Masoin, E., *Bull. de l'Acad. roy. de méd. de Belg.*, 1872, vi, 388.
41. Langendorff, O., *Mitt. a. d. Königsberger physiol. Laboratorium*, 1898, 68.  
Reviewed in *Centralbl. f. d. med. Wissensch.*, 1879, xvii, 406.
42. Johansson, J. E., and Tigerstedt, R., *Mitt. a. d. physiol. Laboratorium in Stockholm*, 1889, vi, No. 8. Reprinted in *Hygiea*, Festband, 1889, No. 5, 1.



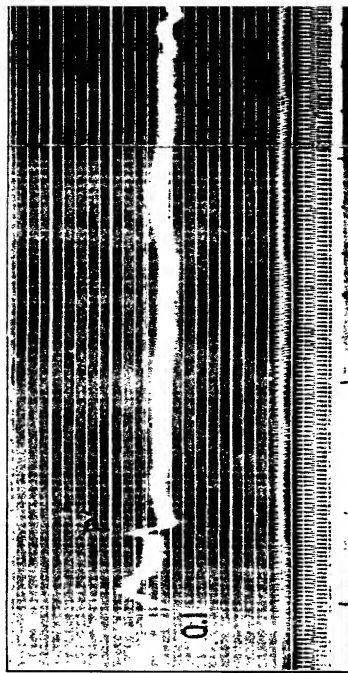
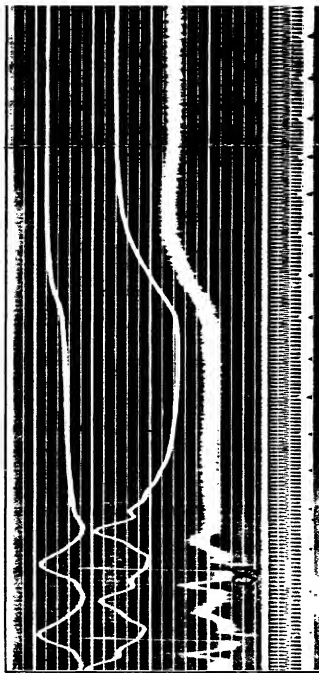


EXPLANATION OF PLATES.

PLATE 6A.

FIG. 1. (Experiment 673.) Stimulation of the right vagus nerve. The curves from above downward are: (1) suspension curve of right auricle, (2) suspension curve of right ventricle, (3) electrocardiogram, (4) signal showing the length of stimulation, (5) time in 0.02 of a second, (6) time in 0.2 of a second. The stimulus applied stopped the whole heart. The P-R time was lengthened slightly, 0.09 to 0.12 of a second.

FIG. 2. (Experiment 666.) Stimulation of the right vagus nerve. No suspension curves are shown here or in the following curves. An auricular contraction (P') occurs shortly after the beginning of stimulation. Three seconds later a complete cycle, and 2.8 seconds later a second cycle escapes; between them occurs one blocked auricular (P') wave. The P-R time in the escaped cycles was lengthened 0.1 to 0.12 and 0.14 of a second.



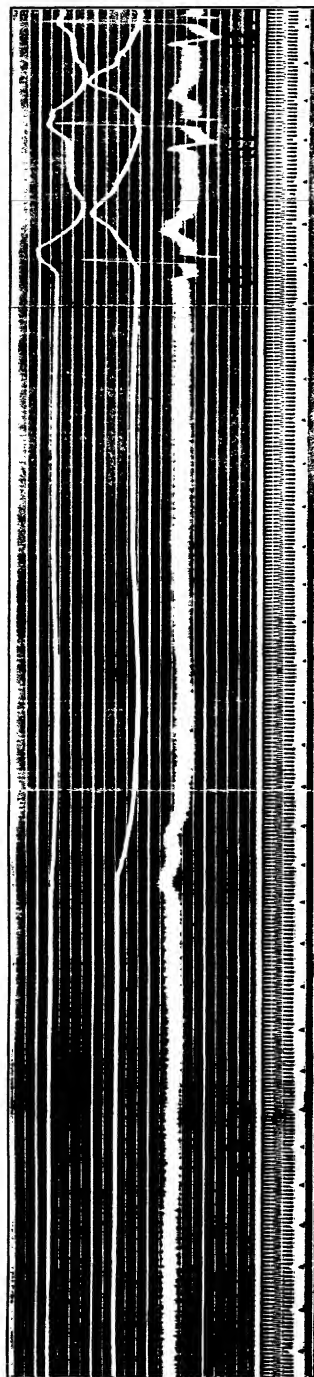


FIG. 1.

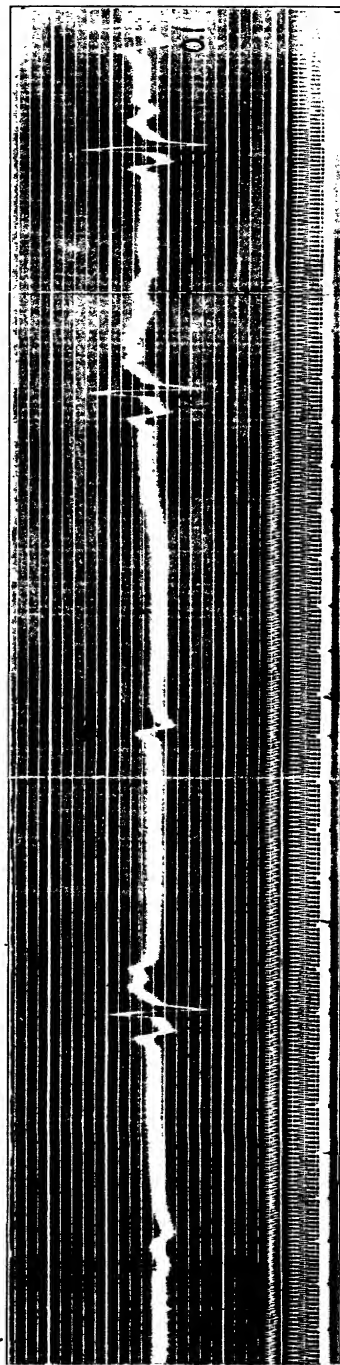


FIG. 2.

PLATE 70.

FIG. 3. (Experiment 66c.) Stimulation of the right vagus nerve. After the cessation of stimulation the first cycle shows a lengthened P-R interval (0.1 to 0.12 of a second), after which a blocked auricular contraction occurs.

FIG. 4. (Experiment 65g.) Stimulation of the right vagus nerve. Shortly after the beginning of stimulation, one blocked auricular (P') contraction occurs. During the period of stimulation two ectopic (right ventricular) contractions are seen. After stimulation the P-R interval is lengthened, 0.11 to 0.18 of a second, and two blocked auricular (P') waves occur.

FIG. 5. (Experiment 66i.) Stimulation of the right vagus nerve. The auricles cease contracting entirely. The complexes representing ventricular contractions are derived from stimuli arising in the wall of the left ventricle. The first two auricular contractions after the cessation of stimulation are blocked. The P-R time is lengthened from 0.11 to 0.14 of a second. It returned to normal in the third cycle after the last one reproduced.

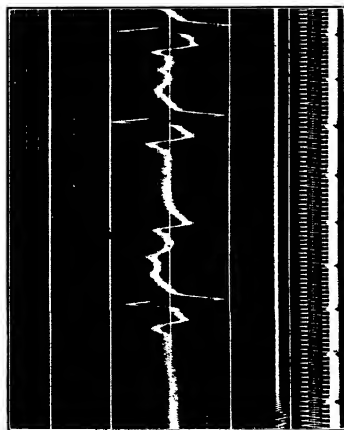
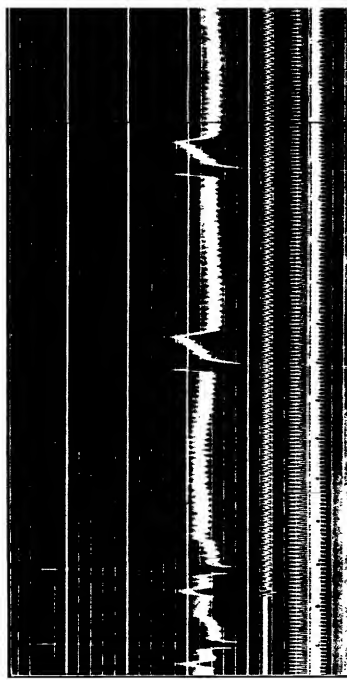


FIG. 3.



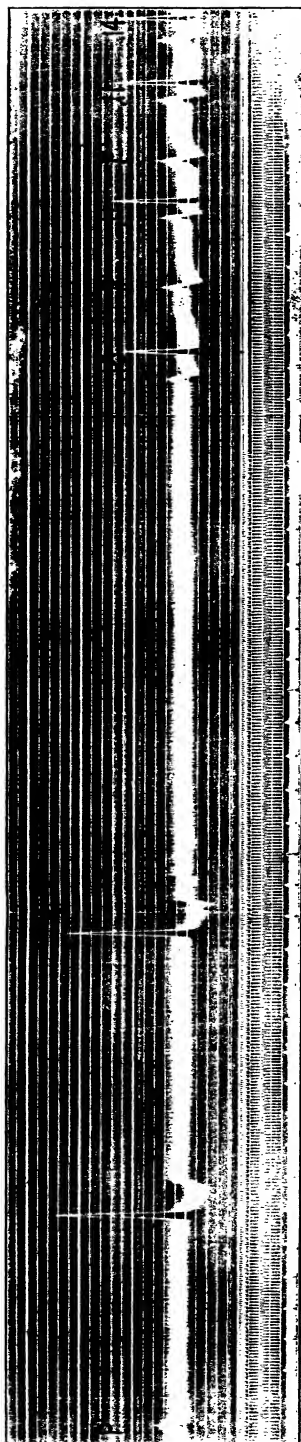


FIG. 4.



FIG. 5.

Contr. Effects of Stimulation of Two Vagus Nerves.

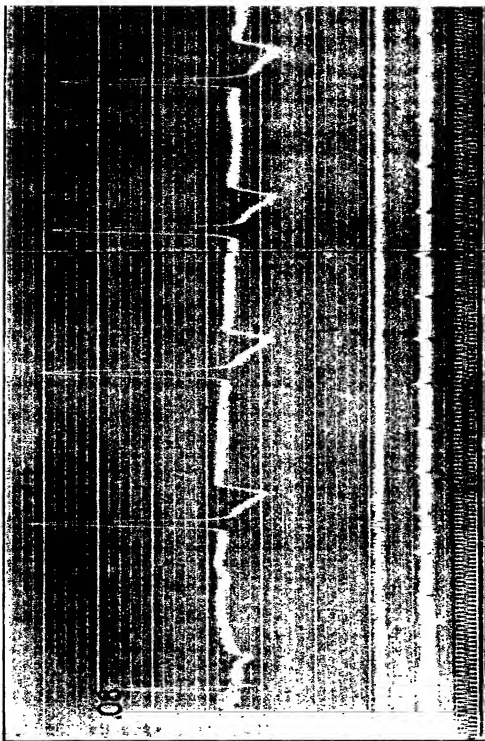
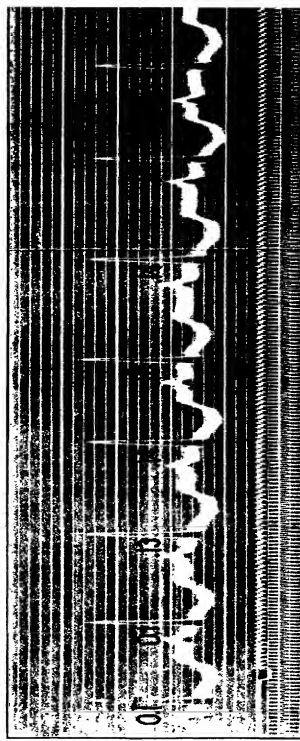


PLATE 71.

FIG. 6.<sup>a</sup> (Experiment 660.) Stimulation of the right vagus nerve. Similar to the effect shown in figure 5, except that the ventricular complexes in this curve originate in the right ventricle. The first three auricular (P) waves after the end of stimulation are dissociated from the ventricular complexes.

FIG. 7. (Experiment 662.) Stimulation of the left vagus nerve. Gradual increase in the length of the P-R time, 0.1 to 0.24 of a second, is shown. The fifth cycle after the last one reproduced has a P-R interval of 0.12 of a second.

<sup>a</sup>In figure 6, on account of the faintness of the original tracings, the R waves of the ventricular complexes have been redrawn in the reproduction.



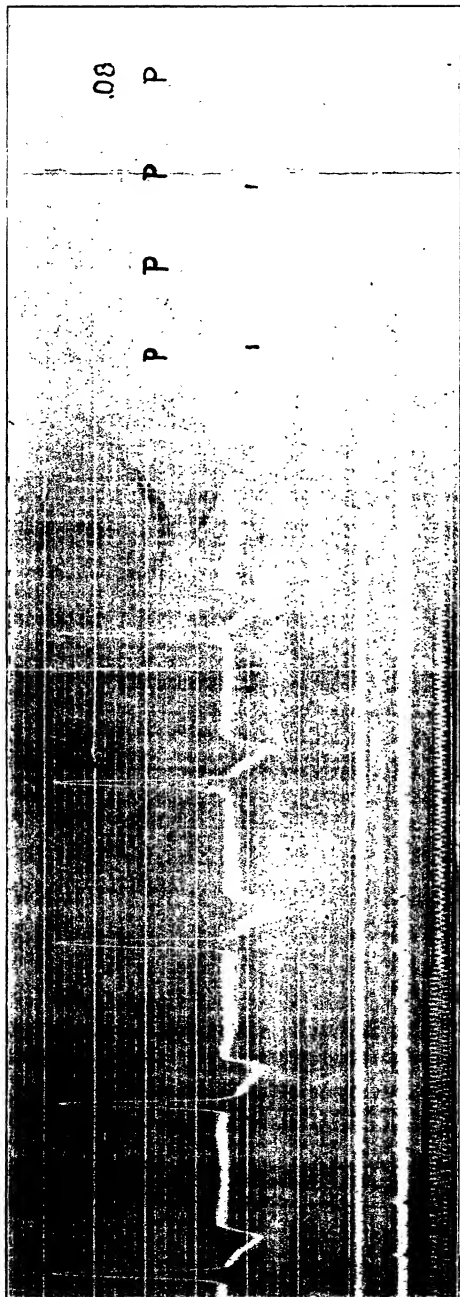


FIG. 6.

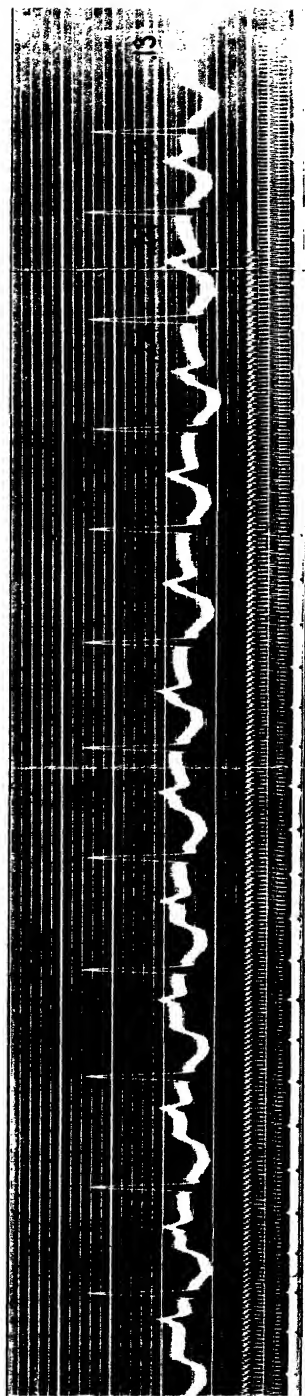


PLATE 72.

FIG. 8. A and B. (Experiment 666.) The second part, B, of this figure is directly continuous with the first portion. The decimials above the electrocardiogram represent the intervals (P-I') between succeeding auricular contractions; the decimials underneath, the P-R interval. The P-R interval in the groups of cycles in figure 8, A, gradually increases until an auricular wave is blocked (P'). In figure 8, B, the last cycle in the group shown has a P-R interval slightly shorter than the cycle immediately preceding.

FIG. 9. (Experiment 673.) Stimulation of the left vagus nerve. Suspension curves as in figure 1. The auricular contractions in the suspension curve gradually become smaller during the eight numbered heats until no elevations are seen on the curve. Ventricular contractions have ceased. The electrical curve shows the P waves, the representatives of auricular contractions, present. After stimulation, the P-R interval is lengthened from 0.09 to 0.12 of a second. An auricular contraction, P', is then blocked and the P-R time lengthens to 0.14 of a second. The fourth cycle after the last one reproduced shows a P-R time of 0.11 of a second.

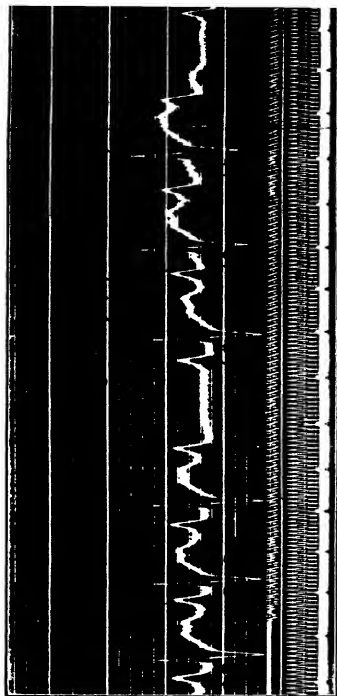
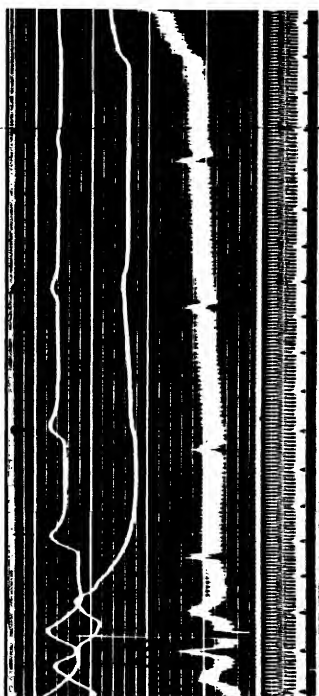


FIG. 8A.



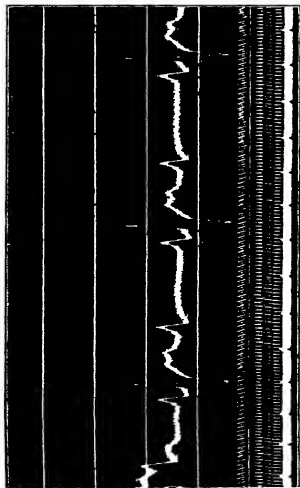


Fig. 8A.

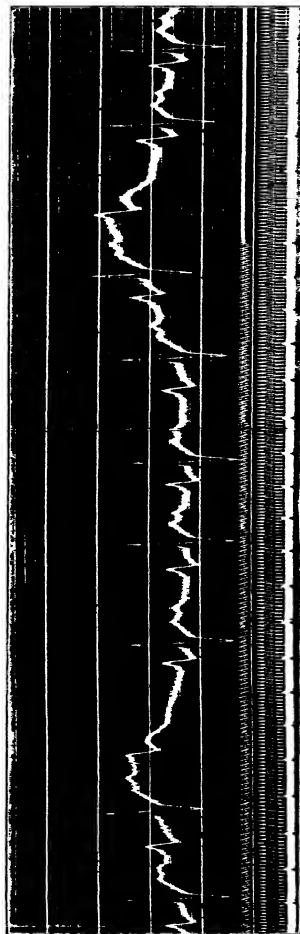


Fig. 8B.

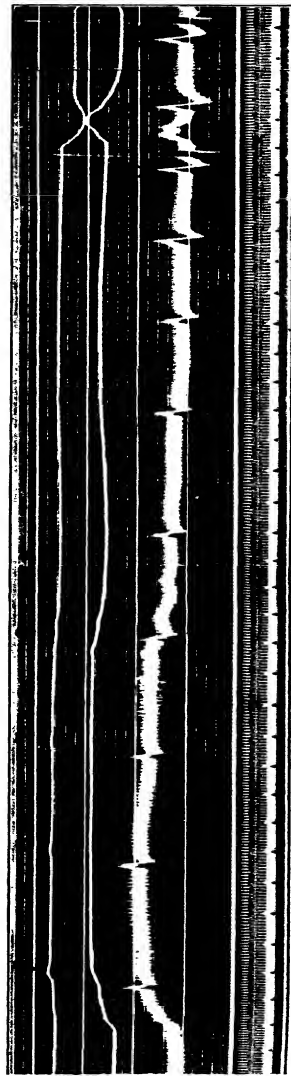


Fig. 9.

Conts: Effects of Stimulation of Two Vagus Nerves.



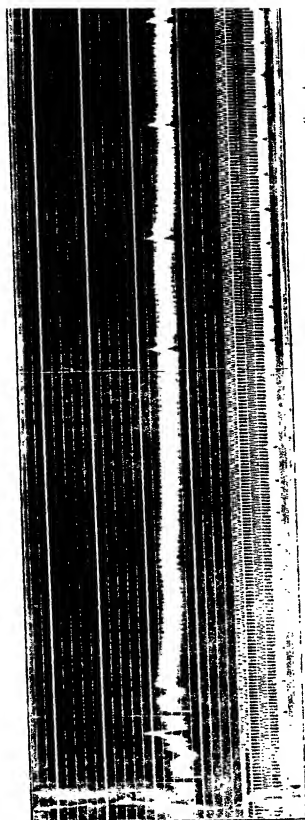


FIG. 10.

FIG. 10. A and B. (Experiment 607.) Stimulation of the left vagus nerve. The two curves are directly continuous. Figure 10, A, shows events like those of figure 9. It differs in that a pause of .23 seconds occurs after the beginning of stimulation and until the auricular contractions begin. Figure 10, B, shows an escaped ectopic (right ventricular) complex. The remainder of the curve shows a 2:1 rhythm. The rest of this curve, which is not reproduced, shows ten additional cycles of a 2:1 rhythm, a sequential cycle, another 2:1 cycle, and finally a sequential rhythm.

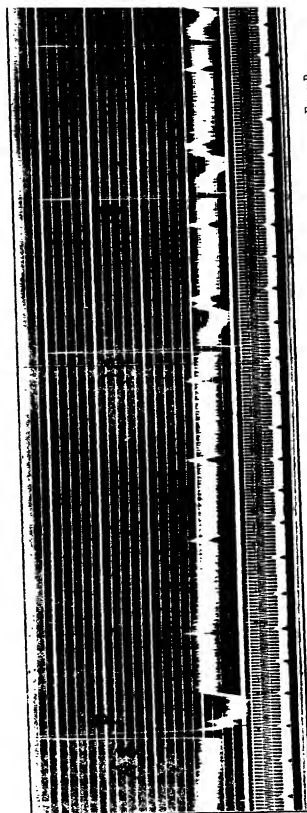


FIG. 10B.

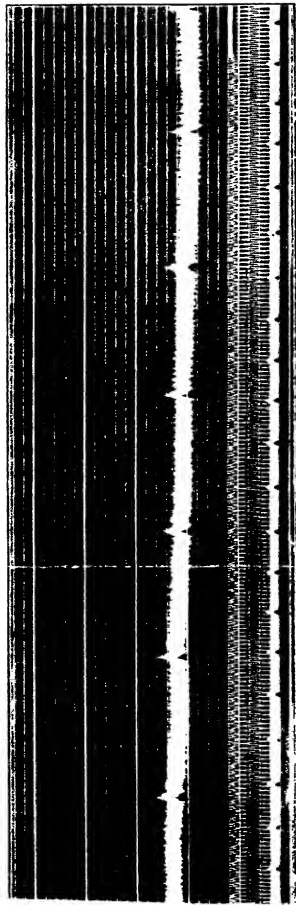


FIG. 10. A and mX.  
The two curves are  
of figure 9. It di-  
of stimulation and  
an escaped ectopic  
shows a 2:1 rhythm  
additional cycles  
finally a sequen-

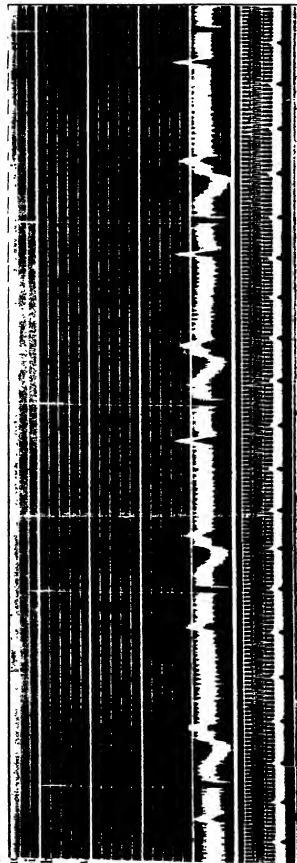


FIG. 10B. Effects of Stimulation of Two Vagus Nerves.

ac. 10B.

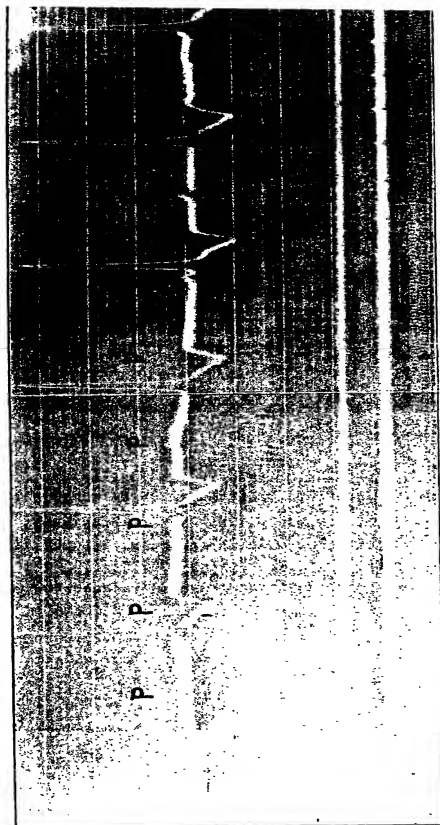


FIG. 11.

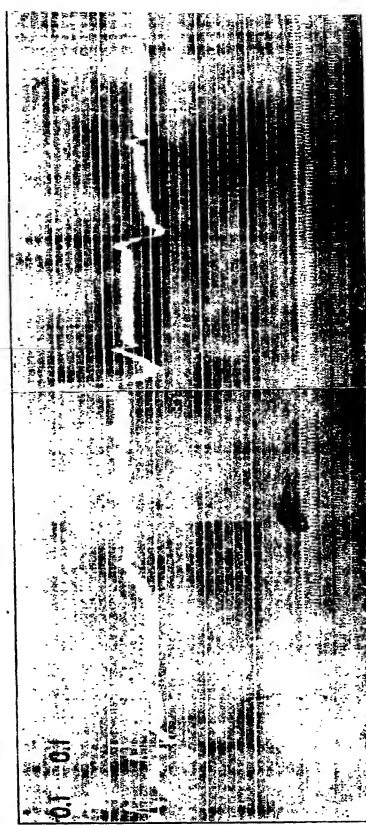


PLATE 74.

FIG. 11.\* (Experiment 634.) Stimulation of the left vagus nerve. The atrial contractions continue. Nonisometric ventricular contractions have ceased. Their place is taken by ectopic ventricular contractions arising in the right ventricle. Atrial and ventricular contractions occur in complete dissociation.

FIG. 12. (Experiment 644.) Stimulation of the left vagus nerve. Atrial contractions continue throughout the period of stimulation. Two ventricular contractions originating in the wall of the right ventricle appear. The P-R time after stimulation is 0.18 of a second. One atrial contraction (P) is then blocked, after which the normal sequence is restored.

\*In figure 11, on account of the faintness of the original tracings, the R waves of the ventricular complexes have been redrawn in the reproduction.

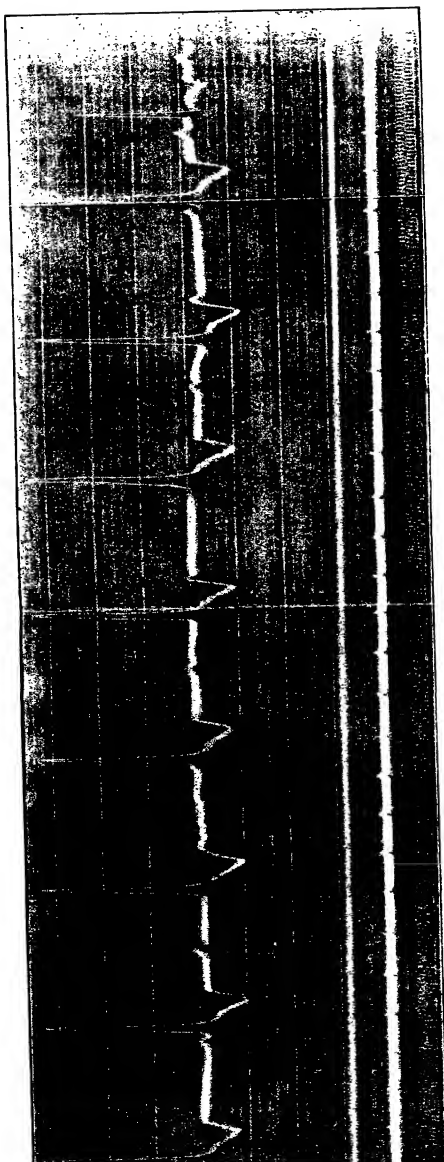


FIG. 11.

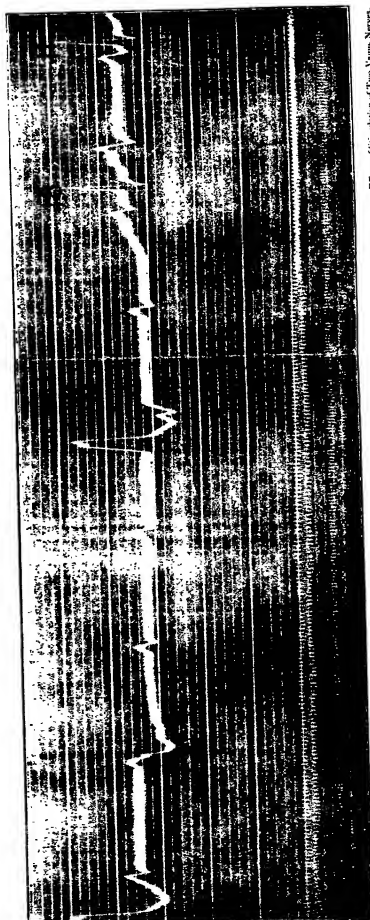


FIG. 12.

Courtesy: Effects of Stimulation of Two Vagus Nerves.

PLATE 75.

FIG. 13. A and B. (Experiment 66.) Stimulation of the left vagus nerve. B is directly continuous with A. In A auricular contractions first ceased. Four are seen toward the end of the curve. Four ventricular contractions are present. These are ectopic (left ventricular) systoles. The last cycle in A and all the cycles in B are sequential and isometric; but are slowed and show a prolonged P-R interval (0.1 to 0.17 of a second).

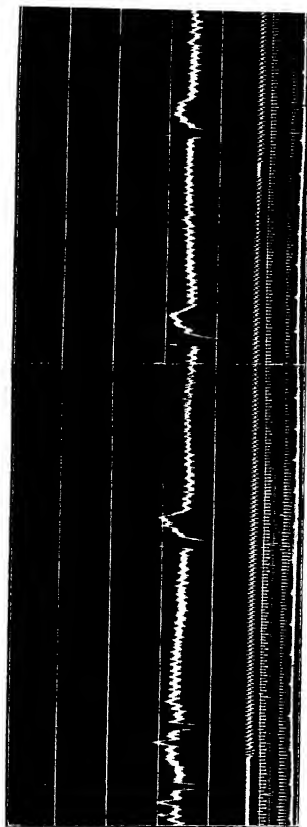


FIG. 13A.

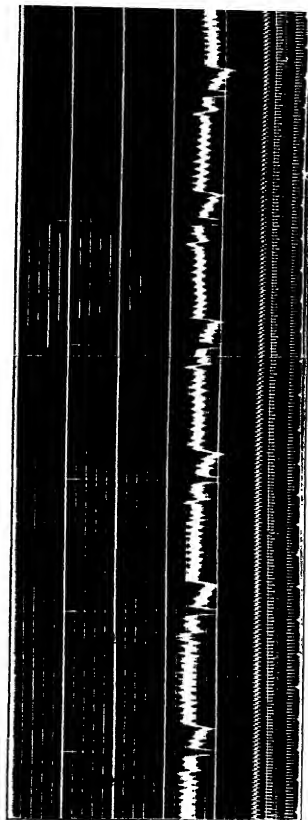


FIG. 13B.

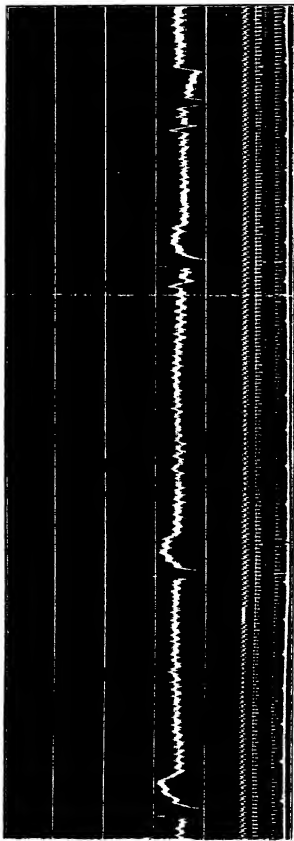


FIG. 13A.

FIG. 13. A and B. (Experiment 661.) S B is directly continuous with A. In A auricular cycles are seen toward the end of the curve. These are ectopic (left ventricular) systoles. cycles in B are sequential and normal, but P-R interval ( $\alpha$  to 0.17 of a second).

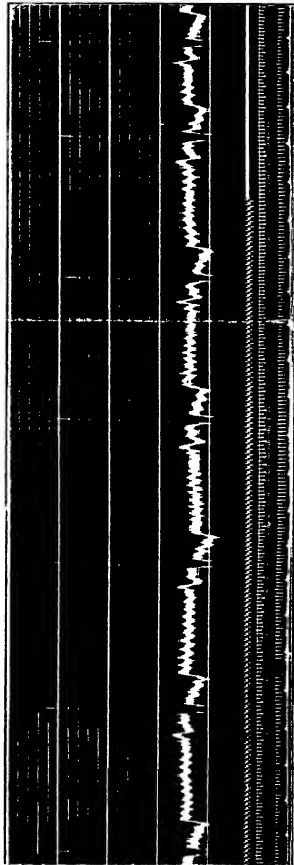


FIG. 13B.

Cover, Effects of Stimulation of Two Vagus Nerves.

PLATE 76

FIG. 14. A and B. (Experiment 62.) Stimulation of the left vagus nerve. The two portions of the curve are not directly continuous. Nine cycles are omitted. The decimals above the electrocardiogram represent the interauricular time; those below, the P-R time. The curve represents an instance of sino-auricular block. For the details the reader is referred to the text (p. 732).

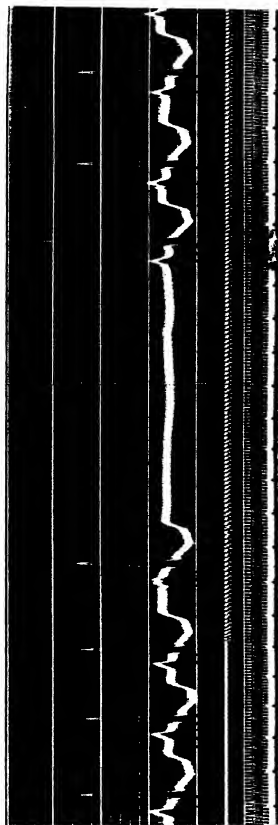


FIG. 14A.

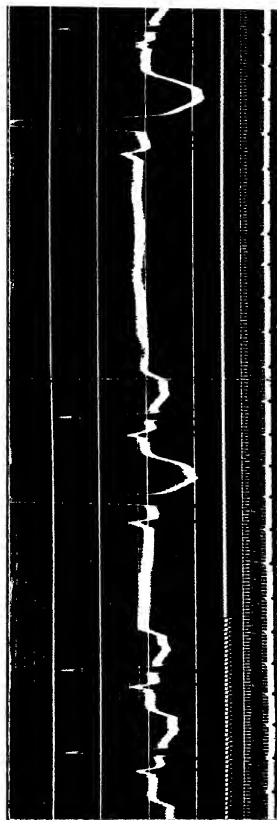


FIG. 14B.

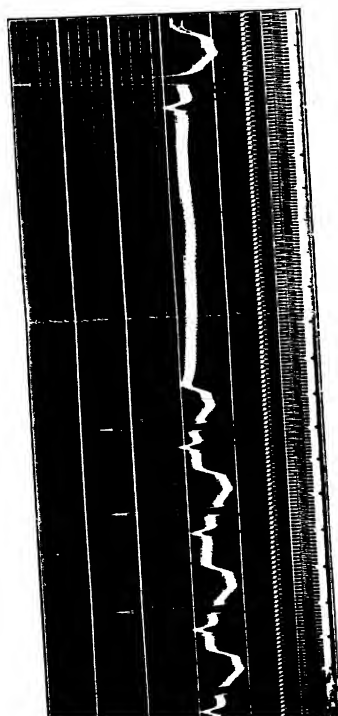


FIG. 14A.

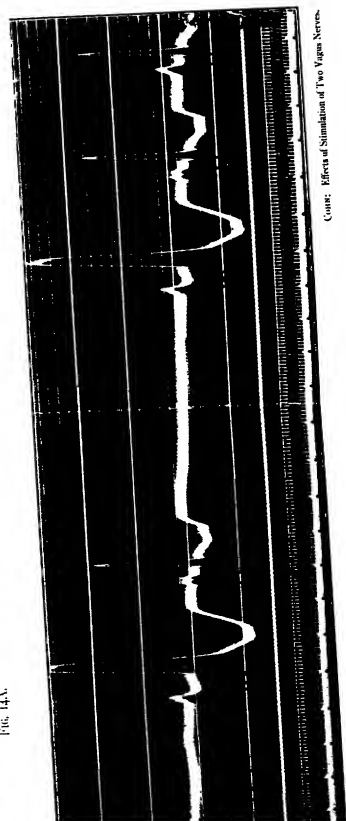


FIG. 14B.

Cont. Effects of Stimulation of Two Vagus Nerves.





43. Harrington, D. W., *Am. Jour. Physiol.*, 1898, i, 383.
44. Eppinger, H., and Rothberger, J., *Ztschr. f. klin. Med.*, 1910, lxx, 1.
45. Eppinger, H., and Stoerk, O., *Ztschr. f. klin. Med.*, 1910, lxxi, 157.
46. Engel, I., *Beitr. z. path. Anat. u. z. allg. Path.*, 1910, xlviii, 499.
47. Wilson, J. G., *Proc. Roy. Soc. London, Series B*, 1909, lxxxix, 151.
48. Kahn, R. H., *Arch. f. d. ges. Physiol.*, 1911, cxl, 627.
49. Kraus, F., and Nicolai, G., *Das Elektrokardiogram*, Leipzig, 1910.
50. von Tabora, D., *Ztschr. f. exper. Path. u. Therap.*, 1906, iii, 499.
51. Keng, Lim Boon, *Jour. Physiol.*, 1893, xiv, 467.
52. von Schumacher, S., *Sitzungsb. d. k. Akad. d. Wissensch., Math.-Naturwiss. Kl., Abt. III*, 1902, cxi, 133.

## THE NATURE AND ORIGIN OF THE NITROGENOUS COMPOUNDS IN THE FECES IN INFANTILISM.\*

BY F. H. MCCRUDDEN AND H. L. FALES.

(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)

Previous studies have shown that the nitrogenous metabolism is on a low plane in infantilism of the type of Herter, and have indicated undernutrition as a possible cause of the failure to grow.<sup>1</sup> As causes of the undernutrition, improper digestion or poor absorption were thought of on account of the large quantities of nitrogen in the feces.<sup>2</sup> The analyses reported in the present paper were carried out for the purpose of determining the nature of the nitrogenous compounds in the feces with the hope that we might be able, with these data, to explain the cause of the loss of nitrogenous material or, at least, to eliminate certain hypotheses which had been made concerning this loss.

For this purpose, determinations of the total nitrogen, and nitrogen in the form of soluble protein, ammonia, purin compounds, bacterial bodies, and amino acids were made. Fairly definite conclusions regarding completeness of digestion, absorption of protein and protein digestion products, and the amount and nature of bacterial action may be reached from these data in so far as these points relate to the problem in hand; namely, whether or not protein is insufficiently, or too rapidly, or completely hydrolyzed, or undergoes changes giving harmful or useless derivatives, or is incompletely absorbed,—all of which possibilities are in accord with the data already obtained.

The feces used were obtained from F. S., one of the patients examined in previous studies, and from E. B., J. P., and M. S.,

\* Received for publication, August 26, 1912.

<sup>1</sup> McCrudden, F. H., and Fales, H. L., *Jour. Exper. Med.*, 1912, xv, 113.

<sup>2</sup> McCrudden, F. H., and Fales, H. L., *ibid.*, 450.

TABLE I.  
*Nitrogen Partition in Feces.*

	Day.	Total nitrogen in gm.	Soluble protein nitrogen.		Ammonia nitrogen.		Purin nitrogen.		Bacterial nitrogen.		Amino nitrogen.	
			In gm.	Per cent.	In gm.	Per cent.	In gm.	Per cent.	In gm.	Per cent.	In gm.	Per cent.
F. S.	1	1.576	—	—	0.080	5.07	0.174	11.03	0.214	13.57	0.053	3.33
	2	1.572	—	—	0.048	3.06	0.230	14.62	0.260	16.53	0.070	4.46
	3	1.444	—	—	0.084	5.82	—	—	0.362	25.05	0.055	3.81
	27	1.344	0.526	39.20	0.088	6.54	0.232	17.30	0.252	18.70	0.1135	8.45
	28	1.184	0.492	41.50	0.060	5.07	0.196	16.50	0.158	13.40	0.0723	6.20
	29	1.064	0.324	30.50	0.072	6.76	0.092	8.65	0.128	12.05	0.0883	8.30
E. B.	1	0.884	0.310	35.10	0.034	3.85	0.114	12.90	0.102	11.55	0.182	20.55
	2	0.660	0.210	31.80	0.034	5.15	0.074	11.20	0.018	2.73	0.120	18.20
	3	0.560	0.102	18.20	0.024	4.28	0.058	10.38	0.046	8.22	0.092	16.40
	14	0.686	0.010	1.46	0.028	4.08	0.094	13.70	0.227	33.10	0.050	7.29
	15	0.624	0.104	16.70	0.029	4.64	0.072	11.50	0.130	20.80	0.043	6.89
	16	0.678	0.058	8.55	0.045	6.64	0.069	10.20	0.198	29.20	0.034	5.02
J. P.	6	1.120	0.294	26.20	0.080	7.14	0.166	14.80	0.294	26.20	0.044	3.93
	7	1.784	0.126	7.06	0.128	7.17	0.220	12.30	0.174	9.76	0.129	7.23
	8	0.557	0.0014	0.25	0.045	8.08	0.000	0.00	—	—	0.066	11.80
	14	1.076	0.0220	2.04	0.060	5.57	0.082	7.62	0.140	13.00	0.032	2.97
	15	0.795	0.0380	4.78	0.021	2.64	0.063	7.92	0.136	17.10	0.037	4.66
	16	0.864	0.1940	22.50	0.058	6.71	0.091	10.50	0.208	24.10	0.045	5.21
M. S. <sup>a</sup>	14	0.717	0.1120	15.60	0.0792	2.68	0.0256	3.58	0.104	14.50	0.0386	5.38
	16	0.966	0.1168	12.10	0.0416	4.31	0.0864	8.95	0.194	20.10	0.0448	4.64
	18											
	18	0.832	0.0430	5.18	0.0216	2.60	0.029	3.49	0.149	17.90	0.046	5.53
	20											

dwarfs of other types without intestinal symptoms, the results from these latter being used for comparison.

Each complete analysis represents one day's feces. The moist fresh stool was ground up with water to a homogeneous emulsion and then made up to a definite volume.

Total nitrogen was determined in 25 c.c. of this suspension by the Kjeldahl method.

Ammonia was determined in 25 c.c. by blowing air through and absorbing the gas in standard acid just as in urine analysis.<sup>a</sup>

Total purins were determined by the method of Krüger and Schittenhelm.<sup>b</sup>

Soluble protein was determined as follows: 200 c.c. of the suspension were made up to 400 c.c., allowed to settle, and then filtered. The protein in the

<sup>a</sup> It was necessary to combine the feces of two days in order to obtain sufficient material.

<sup>a</sup> Folin, O., *Ztschr. f. physiol. Chem.*, 1902-3, xxxvii, 161.

<sup>b</sup> Krüger, M., and Schittenhelm, A., *Ztschr. f. physiol. Chem.*, 1905, xlv, 14.

filtrate was precipitated by boiling after addition of a trace of dilute acetic acid. The nitrogen in the precipitate was determined by the Kjeldahl method.

*Bacterial nitrogen* was determined as follows: 100 c.c. of the suspension were made up to 400 c.c. and allowed to settle. The supernatant liquid was pipetted off and the bacteria precipitated with alcohol. From this point the method of Mattill and Hawk<sup>6</sup> was followed.

All analyses were made in duplicate.

It will be noted that neither the absolute nor the fractional amounts of nitrogen in different forms vary greatly in the three cases (table I).

Both the absolute amount and the percentage of soluble protein are somewhat high. According to Schmidt and Strasburger,<sup>7</sup> increased amounts of soluble protein occur in the feces in various inflammatory and irritative conditions of the gastro-intestinal tract and, as there is sometimes considerable intestinal disturbance in these cases, this disturbance may account for the increase in soluble protein. This increase is not, however, great enough to indicate a loss of food protein through incomplete absorption or decreased hydrolysis. The protein must be hydrolyzed almost, if not completely, to simple products.

The amino acids of the feces are as low as in the controls. They appear to be well absorbed. No abnormal unabsorbable amino acids are formed in appreciable quantities.

Neither the ammonia nitrogen, bacterial nitrogen, nor purin nitrogen are appreciably high, which indicates that putrefactive and fermentative processes are not very active.

The unknown nitrogen compounds left over after subtracting the nitrogen obtained in various forms from the total are variable, but not larger in the case of F. S. than in the other cases.

The findings, then, exclude fairly well the various hypotheses based on improper digestion as possible causes of the faulty development, namely, incomplete hydrolysis of protein, too rapid splitting of proteins, formation of unabsorbable derivatives, and imperfect absorption of normal products, and indicate that the nitrogen of

<sup>6</sup> Mattill, H. A., and Hawk, P. B., *Jour. Exper. Med.*, 1911, xiv, 433. Our centrifuge is so powerful that it easily throws down bacteria. We therefore found it best to modify Hawk's method as indicated.

<sup>7</sup> Schmidt, A., and Strasburger, J., *Die Faeces des Menschen im normalen und krankhaften Zustande*, 3d edition, Berlin, 1910.

the feces occurs in practically the same forms as in normal feces, from which we could conclude that it is presumably of the same origin as in normal feces, that it is chiefly excretory in origin, and that it is not unabsorbed material left over from the food.

The findings are in accord with the belief that the failure to grow in infantilism of the type of Herter is not necessarily associated with any disturbance of the intestinal digestion or absorption of protein material, and to this belief these findings lend considerable weight.

## THE CAUSE OF THE EXCESSIVE CALCIUM EXCRETION THROUGH THE FECES IN INFANTILISM.\*

BY F. H. MCCRUDDEN AND H. L. FALES.

(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)

Many clinical investigators have called attention to cases of failure of development in children and of diseases of the bones in adults following certain forms of intestinal disturbance,<sup>1</sup> and a large proportion of the investigators interested in rickets and various forms of arthritis deformans insists that disturbances in the intestinal canal cause these conditions. But the relationships observed in most of these cases have been rather obscure. The most definite relationship between failure of development and disturbance of bone growth on the one hand, and a disturbance of intestinal function on the other, has been found in the cases of infantilism of the type of Herter. In infantilism of this type there is general retardation in the growth of the body and our observations seem to indicate that this general retardation in growth is secondary to a retardation of the growth of the skeletal system. The bones are frail and thin and fracture easily, and chemical investigation shows disturbances of the calcium metabolism.<sup>2</sup> Complete metabolism experiments show a negative calcium balance in spite of which it is surprising to note that the urine is almost free from calcium, the excess passing off almost entirely in the feces which contain large amounts of this element. In view of the great importance of the subject, this definite finding of a flux of calcium into the intestine in a condition in

\* Received for publication, August 26, 1912.

<sup>1</sup> Schütz, R., *Sammlung klinischer Vorträge*, 1901, No. 318 (*Innere Medizin*, No. 94), 607; *Jahrb. f. Kinderheilk.*, 1905, lxii, 794; *Deutsch. Arch. f. klin. Med.*, 1908, xciv, 125.

Heubner, O., *Jahrb. f. Kinderheilk.*, 1909, lxx, 667.

Koll, E., *Deutsch. Arch. f. klin. Med.*, 1910, c, 487.

<sup>2</sup> McCrudden, F. H., *Jour. Exper. Med.*, 1912, xv, 107; McCrudden, F. H., and Fales, H. L., *ibid.*, 113, 450, 457.

which there is a general retardation of growth and a special disturbance of bone growth seemed worth following further. What is the cause of this flux of calcium?

The analyses reported in the present paper were made with the object of testing three hypotheses relating to the cause of the loss of calcium through the feces. The first is that in this condition there is a primary disturbance of the fat digestion leading to the excretion of large quantities of calcium through the feces in the form of calcium soaps. This hypothesis, suggested by Herter,<sup>3</sup> was based on the finding of large quantities of fats and especially soaps in the feces in certain cases. A second hypothesis is the one to which loss of calcium in rickets is sometimes attributed, namely, that excessive quantities of phosphate in the feces lead to a large loss of calcium phosphate; and a third is that increased intestinal fermentation gives rise to the formation of large quantities of volatile fatty acids which lead to the excretion of calcium in the form of calcium salts of these acids.

In order to determine if the quantities of fatty acid, phosphate, or volatile acid were in great enough excess to account for the losses of calcium, the following substances were determined quantitatively: fats, fatty acids, volatile acids, calcium, magnesium, nitrogen, and sulphur. The following method of calculation was used. The quantity of phosphate necessary to neutralize the magnesium was subtracted from the total phosphate present. The amount of calcium necessary to combine with the remainder of this phosphate was subtracted from the total calcium. The calcium remaining was expressed as centimeters of normal calcium oxide, and from this was subtracted the sum of the fatty and volatile acids expressed in the same terms, which gives the quantity of calcium not combined with phosphate, fatty acid, or volatile acid. This method of calculation is not intended to indicate that the elements are supposed necessarily to be combined in exactly this way in the feces. There are small amounts of chloride present,<sup>4</sup> some of the magnesium is un-

<sup>3</sup> Herter, *On Infantilism from Chronic Intestinal Infection*, New York, 1908.

<sup>4</sup> The amounts of chloride, silicic acid, potassium, sodium, and iron in the feces are small (Schmidt, A., and Strasburger, J., *Die Faeces des Menschen im normalen und krankhaften Zustande*, 3d edition, Berlin, 1910, chapter XVIII).



doubtedly present as soap and some of the fatty acid is probably combined with small amounts of the potassium, sodium, and ammonium present.<sup>5</sup> It is not intended that the method of calculation should be considered as having a general application. But for the purpose of the present tests, namely, to determine if phosphates, fatty acids, or volatile acids are in excess of calcium and magnesium, the method of calculation is correct.

The patients reported as F. S. and F. H. in the table are cases of infantilism of the type of Herter. Wm. McC. is a normal boy of the same age as F. S. E. B. is a case of achondroplasia. J. P. is seventeen years old and has infantilism probably of the type of Lorraine. In the last three of these cases no disturbance of calcium metabolism was observable. They were studied for comparison. The table shows the results.

Column 15 gives the amount of phosphate calculated as phosphorus necessary to combine with magnesium oxide to form magnesium ammonia phosphate. The results in column 16 are obtained by subtracting those of column 15 from those of column 5 and give the amount of phosphorus left after subtracting that combined with magnesium oxide. Column 17 gives the amount of calcium oxide necessary to combine with all the phosphate not combined with magnesium oxide. The results in column 18 are obtained by subtracting the values in column 17 from those in column 3 and give the amount of calcium oxide not combined as phosphate. Columns 12 and 14 give respectively the amounts of fatty acid and volatile acid in terms of cubic centimeters of normal acid. Column 19 gives, in the same terms for comparison, the calcium oxide which is not combined as phosphate. Column 20 is obtained by subtracting the results in column 19 from those in column 14 and represents the quantity of calcium left over, not combined as either phosphate or soap and expressed in terms of cubic centimeters of normal solution. In column 21 these results are expressed in grams of calcium.

The amount of calcium combined as sulphate is more difficult to estimate. The ratio of sulphur to nitrogen (1:10) is about the average ratio in which these elements occur in various protein materials, and so is probably mostly in organic combination; but even if we assume that the sulphur occurs almost entirely as sulphate and is not at all combined with other bases, it would account for very little of the calcium.

<sup>5</sup> Ammonia was determined in scattered specimens but not in large amounts.

TABLE I.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
	Total weight of stool.	Calcium oxide.	Magnesium oxide.	Phosphorus.	Total fat.		Neutral fat.		Fatty acid and soap.		Volatiles.		Fatty acid plus volatile acid.		Phosphate combined with magnesium oxide.		Calcium oxide combined with phosphorus.		Calcium oxide not combined with phosphate or any volatile acids.		Nitrogen.	Sulphur.
					Per cent.	Weight.	Per cent.	Weight.	Per cent.	Weight.	As normal acid in c.c.	As normal acid in c.c.	Fatty acid as normal acid in c.c.		Phosphate combined with magnesium oxide.		Calcium oxide combined with phosphorus.		Calcium oxide not combined with phosphate or any volatile acids.	As normal calcium oxide in c.c.	In gm.	
F. S.	6 days. 231.1	10.26	1.700	7.34	14.80	34.20	3.40	7.86	11.40	26.35	92.80	9.54	102.34		4.35	3.44	6.82	243.6	141.3	3.95	13.52	(1.220) <sup>M</sup>
F. H.	6 days. 117.3	8.785	1.841	(8.14) <sup>*</sup>	16.00	18.38	10.70	12.55	5.35	6.27	22.08	6.69	28.77		4.90	3.86	4.93	176.1	147.3	4.13	5.40	0.676
Wm. McC.	6 days. 122.6	10.19	1.618	4.39	23.20	28.45	5.80	7.11	17.30	21.20	74.60	10.57	85.17	2.84	1.55	1.22	8.97	320.4	235.2	6.59	6.49	0.580
J. P.	10 days. 156.9	5.93	1.466	3.04	11.66	18.30	8.30	13.02	3.36	5.26	18.52	11.76	30.28	2.58	0.46	0.36	5.57	198.9	168.6	4.72	8.76	0.859
E. B.	8 days. 160.4	5.19	1.900	5.60	9.81	15.75	7.20	11.69	2.52	4.04	14.22	14.90	29.12	3.34	2.26	1.78	3.41	121.7	92.6	2.59	8.65	0.917

<sup>\*</sup>This result was calculated from an average of other stools.

It will be seen that the fats, fatty acids, and volatile acids do not vary very much in any of the cases, and, furthermore, that a very large part of the calcium,—about half in all cases,—cannot be combined with either phosphate or volatile fatty acid, but must be combined with substances at present not known. As far as our results go, the calcium seems to be combined in about the same way in the abnormal as in the normal cases, and one might be tempted to say that the disturbance does not lie in the intestine. We are not justified in saying this, however, since the mode in which about half the calcium is combined is still unknown. In view of our present ignorance concerning the form of combination of the bases in normal feces, and until we have more information on this point, it is futile to engage in speculation concerning the form in which the calcium is lost in the feces in these cases of infantilism.

#### SUMMARY.

Analyses of the principal known bases and acids of the feces in cases of infantilism in which excessive amounts of calcium are being lost, and comparison with cases which are normal in this respect, show that the fats, fatty acids, and volatile acids are not high, and lead to the conclusion that the loss of calcium is not secondary to the presence of large quantities of phosphates, fatty acids, or volatile acids.

## STUDIES OF THE INORGANIC METABOLISM IN PNEUMONIA WITH ESPECIAL REFERENCE TO CALCIUM AND MAGNESIUM.\*

By FRANCIS W. PEABODY, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research, New York.)

Most of the investigations which deal with the metabolism of inorganic substances in pneumonia have been devoted to the study of the chlorides. The retention of chlorine, perhaps the most easily demonstrable of the various disturbances of metabolism during fever, is so constant and so pronounced in pneumonia that it has assumed diagnostic importance. That much less attention has been paid to the excretion of the bases probably depends on the fact that somewhat more complicated analytical methods are needed.

The excretion of sodium and potassium was, however, comprehensively studied by Salkowski<sup>1</sup> who found that sodium, as well as chlorine, is retained during fever, but that potassium continues to be excreted. The normal relationship in which the amount of sodium excreted exceeds that of potassium, was shown to be reversed during the febrile stage of several cases of pneumonia. The metabolism of calcium was later investigated by von Moraczewski,<sup>2</sup> who demonstrated by balance experiments that there is a true retention of calcium in pneumonia which is as definite as is that of sodium or of chlorine. At the same time von Moraczewski made a few observations on the excretion of magnesium, in one case for a period of eight days, and in another for two days, and found no evidence of a retention. Beyond this, however, there is little mention in the literature of the metabolism of magnesium during pneumonia.

Tables I and II show the results of analyses for chlorine, calcium, and magnesium in the urine. They are typical examples of sixteen

\* Received for publication, September 27, 1912.

<sup>1</sup> Salkowski, E., *Virchows Arch. f. path. Anat.*, 1871, liii, 209.

<sup>2</sup> von Moraczewski, W., *Virchows Arch. f. path. Anat.*, 1899, clv, 11; *Ztschr. f. klin. Med.*, 1900, xxxix, 44.

TABLE I.

*The Excretion of Chlorine, Calcium, and Magnesium in the Urine in Pneumonia.*

No. of case.	Day of disease.	Temperature F.	Chlorine in gm.	Calcium oxide in gm.	Magnesium oxide in gm.
I	4	105.4	0.50	0.053	0.074
	5	102.4	0.88	0.023	0.383
	6	105.0	0.83	0.022	0.127
	7	99.4	0.73	0.040	0.260
	8	99.6	0.72	0.136	0.181
	9	98.8	1.45	0.161	0.101
	10	98.0	4.49	0.258	0.156
	11	98.2	5.93	0.408	0.225
	12	98.0	10.05	0.675	0.274
	13	97.6	9.33	0.686	0.243
II	9	99.4	3.95	0.017	0.455
	10	99.4	—	—	—
	11	98.8	4.49	0.053	0.467
	12	99.0	5.04	0.022	0.484
	13	97.8	7.71	0.340	0.364
	14	98.0	6.96	0.290	0.271
	15	98.0	3.34	0.174	0.165
	16	97.8	11.64	0.414	0.347
	17	98.6	5.98	0.312	0.282
IV	8	104.8	1.28	0.036	0.454
	9	104.0	1.18	0.023	0.240
	10	101.8	0.83	0.017	0.225
	11	101.2	1.00	0.032	0.133
	12	102.0	0.55	0.021	0.162
	13	100.8	0.99	—	—
	14	101.2	1.75	0.059	0.151
	15	102.0	3.81	0.109	0.141
	16	100.6	3.76	0.152	0.165
	17	100.4	4.73	0.201	0.139
V	11	100.9	0.58	0.013	—
	12	100.5	0.75	0.039	0.307
	14	100.6	1.81	0.031	0.203
	18	99.0	5.74	0.076	—
VI	10	103.3	0.48	0.029	0.312
	13	101.7	1.00	0.019	0.123
	14	101.0	0.93	0.026	0.180
	18	99.8	6.43	0.099	0.146
IX	6	104.2	—	0.020	0.099
	7	99.0	—	0.017	0.215
	11	98.8	—	0.169	0.128
	12	98.4	—	0.223	0.205
	13	98.0	—	0.332	0.223
	14	98.0	—	0.344	0.194
X	5	104.5	0.13	0.008	0.008
	7	104.0	0.24	0.020	0.013
	8	103.4	0.37	0.041	0.051
	9	101.5	0.34	0.027	0.302

TABLE I.—*Continued.*

No. of case.	Day of disease.	Temperature F.	Chlorine in gm.	Calcium oxide in gm.	Magnesium oxide in gm.
X	11	99.0	1.13	0.017	0.313
	12	98.4	8.51	0.071	0.222
	13	98.0	14.03	0.084	0.181
	14	98.4	8.58	0.169	0.210
	15	98.0	15.94	0.208	0.226
	16	98.0	11.78	0.273	0.263
	17	98.0	13.38	0.273	0.251
XI	9	99.6	0.99	0.032	—
	10	99.4	3.85	0.043	0.169
	11	99.4	6.22	0.059	0.153
	12	98.4	7.43	0.104	0.215
	13	98.4	9.59	0.161	0.189
	14	98.4	8.08	0.132	0.213
	16	98.6	23.38	0.431	0.221
XII	3	104.4	0.62	0.018	0.214
	4	104.2	1.56	0.020	0.367
	5	104.2	3.28	0.022	0.209
	6	104.0	4.29	0.021	0.341
	7	103.0	3.08	0.050	0.281
	8	98.6	2.92	0.098	0.236
	9	98.2	7.29	0.136	0.042
	10	98.4	15.86	0.351	0.082
	11	98.8	17.71	0.321	0.250
	12	99.4	12.48	0.206	0.192

Case XII received 10 gm. of sodium chloride daily. Case VII received 2 gm., cases VIII and XIV, 4 gm., and cases XIII and XV, 6 gm. of calcium lactate daily in addition to their diet. (For cases XIV and XV, see table II.)

cases of pneumonia in which rather extensive studies of the urine were made. They show, in agreement with von Moraczewski, that there is constantly a retention of calcium during the height of the disease. This calcium retention persists often for some days after the temperature has fallen to normal, and the excretion of calcium begins almost simultaneously with the excretion of chlorine. The figures for magnesium are also remarkably uniform and indicate clearly that this base is not retained, but that it is excreted normally in both febrile and afebrile stages of the disease. In many instances, indeed, in spite of the restricted diet, there is definitely more magnesium excreted during the fever than there is after the fever has passed. The only case in which there was any evidence of a retention of magnesium was seen in case X. In this case, however, on the three days on which low quantities of magnesium were

TABLE II.

No. of case.	Day of disease.	Temperature F.	Chlorine in urine in gm.	Calcium oxide in urine in gm.	Magnesium oxide in urine in gm.	Calcium oxide in feces in gm.	Magnesium oxide in feces in gm.	Approximate calcium oxide intake in gm.	Approximate magnesium oxide intake in gm.
XIV	4	104.8	0.56	0.024	0.100	—	—	—	—
	5	104.3	0.61	0.028	0.183	—	—	—	—
	6	104.0	0.41	0.011	0.213	—	—	—	0.22
	7	104.6	0.27	0.014	0.151	2.790	0.334	3.0	0.21
	8	100.4	0.13	0.029	0.264	4.091	0.493	3.0	0.20
	9	100.6	—	—	—	—	—	—	—
	10	100.6	2.82	0.050	0.313	2.642	0.355	2.6	—
	11	100.0	3.49	0.140	0.256	1.015	0.358	3.0	—
	12	100.0	3.61	0.162	0.202	2.681	0.418	3.2	—
	13	99.6	7.62	0.305	0.233	3.517	0.582	3.2	—
	14	99.0	9.02	0.267	0.175	2.548	0.378	3.2	—
	15	99.6	11.30	0.315	0.194	1.223	0.213	3.4	—
	16	98.6	10.27	0.374	0.249	1.413	0.261	3.0	—
	17	98.6	—	—	—	2.064	0.330	—	—
XV	5	105.5	}	0.1793	0.538	{ —	—	3.5	0.20
	6	105.4		—	—	1.269	0.645	4.7	0.32
	7	105.9		0.162	0.152	1.272	0.234	5.9	0.44
	8	105.8		0.109	0.155	3.475	0.433	4.3	0.28
	9	105.7		0.084	—	2.273	0.480	4.1	0.27
	10	104.6		0.062	—	1.765	0.213	4.5	0.30
	11	104.2		—	—	2.258	0.261	—	—

excreted, the urine contained amounts of albumen which were much larger than are ordinarily found in pneumonia. It is probable, then, that the retention of magnesium depended here upon a nephritis, which Rumpf<sup>a</sup> has shown may interfere with the excretion of any of the inorganic bases. This view is borne out by the fact that the sudden rise in the magnesium excretion coincided with a marked drop of the albumen in the urine. The demonstration that the excretion of magnesium goes on normally in pneumonia during the period in which calcium and chlorine are retained, is a point of evidence against the frequently recurring suggestion that chloride retention depends on a kidney lesion.

In order to be certain that a calcium starvation was not giving rise to a urinary picture which might simulate a retention of calcium, several patients received calcium lactate (four to six grams daily) in addition to the calcium in their regular diet. As the diet, especially in the febrile period, contained considerable amounts of milk, the daily calcium intake was a liberal one. That there is no

<sup>a</sup> Rumpf, Th., *München. med. Wchnschr.*, 1905, lii, 393.

especial difference in the absorption of calcium from the intestine in the febrile and afebrile periods is shown by cases XIV and XV, in which the stools were analyzed for calcium and magnesium. The food was not analyzed, but inasmuch as during the febrile period the patients were practically on a milk diet and as all food was measured with great care, it is possible to calculate fairly closely the intake of calcium and magnesium. Unfortunately in both these cases specimens of urine were lost, so that the results for certain days had to be thrown out, but in spite of this they both show that the excretion of magnesium in the urine and feces greatly exceeded the intake. There is thus a loss of magnesium to the body during the febrile period. The relation between the amount of magnesium in the feces and that in the urine was about the same in the two periods.

The disturbance of the urinary excretion of inorganic substances in pneumonia is, therefore, profound. On the one hand, there is a retention in the body of sodium, calcium, and chlorine. On the other hand, there is a normal or excessive excretion of potassium and magnesium. The normal quantitative relation of sodium to potassium and of calcium to magnesium is reversed in the febrile urine. It is interesting that sodium and calcium, the retained bases, occur in larger proportions than do magnesium and potassium in body fluids. In muscle the ratio is reversed. The excessive excretion of potassium and magnesium during fever thus falls in line with the high nitrogen excretion, and all three undoubtedly represent the increased tissue catabolism. This general type of metabolism is undoubtedly characteristic of fever or infection. It is not to be supposed that it is in any way specific for pneumonia, but the sharp change from the febrile to the afebrile period in lobar pneumonia gives an unusually good opportunity for studying the process.

#### ANALYSES OF THE BLOOD DURING AND AFTER THE FEBRILE PERIOD.

In order to study the mechanism of the retention of inorganic substances more directly, the blood was analyzed during the period of retention and again after excretion had begun. The quantitative estimation of chlorine in the blood is comparatively simple and, as the amount of chlorine present is considerable, the errors are small



and the results more reliable than in the case of calcium. A number of isolated observations have already been made on the chlorine content of the blood in pneumonia. Thus Biernacki,<sup>4</sup> Jarisch,<sup>5</sup> Runeberg,<sup>6</sup> Laudenheimer,<sup>7</sup> and Schenk<sup>8</sup> have each reported one or two cases in which they found a decrease in the chlorine content during fever, and in Schenk's case there was a rise during convalescence. Vanderhoof<sup>9</sup> gives two cases with figures for the febrile period which are slightly below his average. One of these showed a fall on the day after the crisis. von Moraczewski<sup>10</sup> has made a more thorough study and has reported eight cases, most of which showed a diminution of chlorine in the blood during the fever. In three cases a second examination was made after the crisis and in two a rise was noted, while in one there was a fall in chlorine. Santini<sup>11</sup> states that the chlorine content of the blood is higher during the febrile period than afterward. Hutchison<sup>12</sup> analyzed pleuritic effusions from cases of pneumonia and found that they contained less chlorine than effusions in other diseases.

To obtain as complete information as possible about the chlorine content of the blood during fever and about the relation of changes in the blood to changes in the urinary excretion, eight cases were studied and twenty-three observations were made (table III). The blood was taken during the period of chlorine retention, and in five cases again shortly after chlorine excretion had begun. Several of the cases were given sodium chloride by mouth to avoid having a chlorine starvation. Eight control observations on one normal adult and four syphilitics (without fever) gave an average normal chlorine content of 0.276 of a gram per 100 grams of blood. In only one case of uncomplicated pneumonia was this figure approached during the first period. The other cases show a much

<sup>4</sup> Biernacki, E., *Ztschr. f. klin. Med.*, 1894, xxiv, 460.

<sup>5</sup> Jarisch, A., abstracted in *Jahresb. ü. d. Leistung. in d. ges. Med.*, 1877, i, 165.

<sup>6</sup> Runeberg, J. W., *Deutsch. Arch. f. klin. Med.*, 1884, xxxv, 266.

<sup>7</sup> Laudenheimer, R., *Ztschr. f. klin. Med.*, 1892, xxi, 513.

<sup>8</sup> Schenk, S. L., abstracted in *Jahresb. ü. d. Leistung. in d. ges. Med.*, 1873, vii, 129.

<sup>9</sup> Vanderhoof, D., *Jour. Am. Med. Assn.*, 1908, li, 478.

<sup>10</sup> von Moraczewski, W., *Virchows Arch. f. path. Anat.*, 1896, cxlvi, 424.

<sup>11</sup> Santini, A., *Riforma med.*, 1903, xix, 477.

<sup>12</sup> Hutchison, R., *Jour. Path. and Bacteriol.*, 1898, v, 406.

lower chlorine content of the blood during chlorine retention, and all cases examined show a rise of the chlorine in the blood when the chlorine begins to be excreted in the urine. The third observa-

TABLE III.

No. of case.	Date.	Temperature F.	Chlorine per 100 gm. of blood.		Chlorine excreted in urine for 24 hours.	Remarks.
			During chloride retention.	During chloride excretion.		
I	Mar. 19	104.4	0.226	—	—	Three hours before death.
II	Mar. 20	104.0	0.266	—	0.00	Blood taken 1½ hours after eating 10 gm. of sodium chloride.
	Mar. 21	103.3	0.265	—	0.35	
	Mar. 22	102.2	0.276	—	0.48	
	Mar. 25	99.0	0.276	—	1.00	
	Mar. 31	99.5	—	0.285	4.39	
III	Mar. 21	99.5	0.253	—	0.53	Crisis during preceding night.
	Mar. 22	99.0	0.252	—	0.58	
	Mar. 24	98.0	—	0.291	4.70	
IV	Mar. 23	101.9	0.254	—	0.00	
	Mar. 24	99.2	0.254	—	1.06	
	Apr. 3	Normal	—	0.293	5.74	
	Apr. 4	Normal	—	0.285	—	
	Apr. 13	Normal	—	0.273	—	
V	Apr. 5	101.6	0.256	—	0.10	Received 5 gm. of sodium chloride twice daily, beginning April 5.
	Apr. 6	102.0	0.245	—	0.08	Developed acute nephritis.
	Apr. 15	99.6	0.274	—	0.70	
VI	Apr. 18	102.0	0.224	—	0.00	Received 5 gm. of sodium chloride twice daily, beginning April 18.
	Apr. 20	99.8	—	0.277	4.27	
	Apr. 22	98.4	—	0.261	3.61	
VII	Apr. 25	102.5	0.256	—	0.38	
	May 1	Normal	—	0.276	2.11	
VIII	Mar. 28	103.0	0.245	—	—	Received 5 gm. of sodium chloride on the evening before, and 5 gm. of sodium chloride three hours before blood was taken.

tion in case V shows a rise of chlorine in the blood without a corresponding rise in the urine, but at this time a severe acute nephritis had set in, so that the blood finding is characteristic for that condition. The results obtained thus agree with the bulk of previous

evidence, and make it certain that chlorine retention in pneumonia is associated with a low chlorine content of the blood.

There is very little available evidence as to the calcium and magnesium content of the blood in pneumonia. Both of these bases are present in such small quantities that a large amount of blood must be taken for analysis, and even then the error is liable to be considerable. von Moraczewski<sup>13</sup> gives the results of analyses for calcium of two specimens of blood from persons who had died of pneumonia. These contained 0.003 and 0.002 of a gram of calcium per 100 grams blood. In another paper<sup>14</sup> he reports several analyses for calcium, but they were made in specimens of only seven to fifteen cubic centimeters of blood, and the precipitates were so small that he puts no value on the results. Jarisch<sup>15</sup> found a higher percentage of calcium in the blood of a patient with pneumonia than in a specimen of normal blood. Nicholls,<sup>16</sup> using the method of Bell and Hick,<sup>17</sup> reports finding a low calcium index in pneumonia.

Table IV shows the results for calcium and magnesium obtained in the analysis of blood from seven patients during and after the period of calcium retention. Each determination was made in a specimen of 100 to 125 cubic centimeters of blood, but the figures for both bases are so small that the results are much less satisfactory than was the case with chlorine. In the two cases, however, in which blood was taken both during and after the period of calcium retention, the amount of calcium in the blood was lower during retention than it was after excretion had begun. The seven individual analyses during the period of retention show considerable variations, but the average is lower than the average of the four observations made in the normal period. It is certain, then, that there is no accumulation of calcium in the blood, as often occurs in nephritis.<sup>18</sup> The results suggest that in pneumonia the mechanism of calcium retention is the same as that of chlorine, and that their retention bears no relation to kidney lesions. Since it is generally

<sup>13</sup> von Moraczewski, W., *Ztschr. f. physiol. Chem.*, 1897, xxiii, 483.

<sup>14</sup> von Moraczewski, W., *Virchows Arch. f. path. Anat.*, 1896, cxlvi, 424.

<sup>15</sup> Jarisch, A., *loc. cit.*

<sup>16</sup> Nicholls, *Proc. and Tr. Royal Soc. Canada*, 1910, ser. 3, iv, sec. iv, 85.

<sup>17</sup> Bell, W. B., and Hick, P., *Brit. Med. Jour.*, 1909, i, 592.

<sup>18</sup> Dennstedt, M., and Rumpf, Th., *Ztschr. f. klin. Med.*, 1906, lviii, 84.

TABLE IV.  
*The Calcium and Magnesium Content of the Blood in Pneumonia.*

No. of case.	Date.	Temperature F.	Calcium oxide in 100 gm. of blood.		Calcium oxide excreted in urine for 24 hours.	Magnesium oxide in 100 gm. of blood.		Magnesium oxide excreted in urine for 24 hours.	Remarks.
			During calcium retention.	After calcium excretion had begun.		During calcium retention.	After calcium excretion had begun.		
I	Mar. 18	Normal	—	0.0072	—	—	0.0068	—	Temperature normal for 17 days.
II	Mar. 16	104.5	0.0065	—	0.024	0.0064	—	0.100	Received 4 gm. of calcium lactate daily.
	Mar. 19	104.0	0.0064	—	0.011	0.0058	—	0.151	
	Mar. 26	Normal	—	0.0092	0.267	—	0.0078	0.175	
	Mar. 28	Normal	—	0.0068	0.374	—	0.0076	0.249	
III	Mar. 17	104.0	0.0077	—	0.020	0.0074	—	0.099	Received 2 gm. of calcium lactate daily.
	Mar. 23	Normal	—	0.0092	0.223	—	0.0094	0.205	
IV	Mar. 28	103.0	0.0074	—	0.013	0.0059	—	0.191	Received 4 gm. of calcium lactate daily.
V	Apr. 9	105.3	0.0078	—	0.010	0.0057	—	0.075	Received 6 gm. of calcium lactate daily.
VI	Apr. 11	102.0	0.0080	—	0.053	—	—	0.142	Received 4 gm. of calcium lactate daily.
VII	Apr. 7	105.5	0.0073	—	—	0.0072	—	—	Urines for April 6 and 7 mixed. The 48 hour specimen contains 0.179 gm. of calcium oxide and 0.538 gm. of magnesium oxide. Received 6 gm. of calcium lactate daily.

conceded that the sodium that is retained is combined with chlorine, it is safe to conclude that the excretion of calcium, sodium, and chlorine is hindered by the same cause.

In the blood the amount of magnesium, like that of calcium, varies somewhat from case to case, but in the two instances in which the blood was examined during the febrile, and later during the afebrile period, the quantity of magnesium was found to rise after the fall of fever. Similarly, the average of all the examinations made during the febrile period is slightly, but distinctly lower than those made in the afebrile period. The condition is thus exactly similar to that found in the analyses for calcium, and this is more interesting since, under the circumstances, magnesium is excreted in large amounts, while calcium is scarcely excreted at all. In his case of pneumonia Jarisch<sup>19</sup> also found no increase in the magnesium over that in his control. Thus the evidence as to the amount of calcium and magnesium in the blood and their excretion by the kidney during fever shows that the essential factor in explaining excretion and retention is neither the kidney nor the blood, but that in order to find an explanation one must go back a step farther to the tissues themselves. In the case of magnesium the tissues continually give up a large amount of the base to the blood and this is readily and normally excreted by the kidney, so that there is no accumulation in the blood. Calcium, on the other hand, is retained in them and given off to the blood only in small amounts. To explain the fact that the magnesium content of the blood is lower during fever than afterwards is difficult, but one may perhaps suggest that at a time when sodium and calcium are being excreted in very small amounts, the kidney is able to excrete magnesium and potassium even more easily than normally.

#### THE RETENTION OF SALTS IN THE TISSUES.

It would seem *a priori* comparatively easy to demonstrate the presence of these retained substances in the tissues. Many investigations have been made to discover in what tissues retention takes place, but although differences may be found between the chlorine content of organs from patients with pneumonia and from patients

<sup>19</sup> Jarisch, A., *loc. cit.*

dying of other diseases, these are usually not marked enough to prove that any one organ acts as a storehouse. While more chlorine is found in the pneumonic lung than in the normal lung, the difference by no means accounts for the degree of retention in many cases. As examples of the amount of chlorine that may be retained in the pneumonic exudate, two cases may be cited in which one lung was practically completely solidified and the other quite free of signs of pneumonia. In the first case the normal lung contained 1.022 grams of chlorine, and the pneumonic lung 2.838 grams. The second patient had received ten grams of sodium chloride in the two days before death and had excreted practically none. The normal lung contained 0.715 of a gram of chlorine, and the solidified lung 2.916 grams. Thus in neither case was much more than two grams of the retained chlorine accounted for in the lungs. The majority of observers have found even less retention in the other organs. The small amount of chlorine found in the tissues is the more surprising when one realizes the extent to which retention may be carried. One patient received ten grams of sodium chloride daily for six days, and during the same period excreted less than sixteen grams.

On account of the work of Padtberg,<sup>20</sup> who showed by intravenous injections of sodium chloride in dogs that the skin is a storehouse for chlorine, it seemed worth while to determine the chlorine content of the skin in pneumonia, especially as there are very few analyses of skin on record. The skin of two control cases contained 0.196 per cent. and 0.178 per cent. of chlorine respectively. Skin from three patients who died of pneumonia contained respectively 0.169 per cent., 0.193 per cent., and 0.194 per cent. of chlorine. The difference between the controls and the pneumonia cases is thus too small to be of any importance. Santini<sup>21</sup> also found no evidence of any especial retention in the skin.

The evidence at present points to the fact that the retained salts are deposited in the tissues throughout the body, and that the percentage increase in any one organ is so slight that it is not brought out strikingly by the chemical analysis.

<sup>20</sup> Padtberg, J. H., *Arch. f. exper. Path. u. Pharmacol.*, 1910, lxiii, 60.

<sup>21</sup> Santini, A., *loc. cit.*

## METHODS.

The chlorine in the urine was titrated according to the method of Harvey<sup>22</sup>. Calcium and magnesium in the urine were determined by McCrudden's<sup>23</sup> method.

In the analyses for chlorine in the blood, about 15 to 20 c.c. of blood were taken into oxalate solution. The coagulable proteid was precipitated by adding 20 per cent. magnesium sulphate and 1 per cent. acetic acid, boiling, neutralizing with 1 per cent. sodium hydrate, and boiling again. The chlorine was titrated in the filtrate. Calcium and magnesium were determined in samples of 100 to 130 c.c. of blood. The blood was ashed in a platinum crucible, according to Bunge's<sup>24</sup> method, and the bases were determined by McCrudden's method. Tissues were ashed similarly, and the chlorine was titrated.

I am greatly indebted to Miss Vinograd for much assistance in the analytical work.

## CONCLUSIONS.

During pneumonia the metabolism of inorganic substances deviates markedly from the normal. While chlorine, sodium, and calcium are retained in the body, potassium and magnesium are excreted normally or in excess. Two cases showed a definite loss of magnesium to the body in the febrile period.

During the period of retention the chlorine content of the blood is distinctly lower than normally, the calcium content is apparently slightly lower, and the magnesium content tends also to be a little lower.

The skin is shown to play no special part in the chlorine retention. Since no organ or organs have been shown to store up large amounts of the retained substances, it is probable that they are spread diffusely throughout the body.

<sup>22</sup> Harvey, S. C., *Arch. Int. Med.*, 1910, vi, 12.

<sup>23</sup> McCrudden, F. H., *Jour. Biol. Chem.*, 1911-12, x, 187.

<sup>24</sup> Bunge, G., *Ztschr. f. Biol.*, 1876, xii, 191.

## ABSORPTION OF ARSENIC FOLLOWING INTRA- MUSCULAR INJECTIONS OF SALVARSAN AND NEOSALVARSAN.\*

By HOMER F. SWIFT, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research, New York.)

### PLATE 5.

The introduction of neosalvarsan has reopened the question of intramuscular injections of organic arsenic preparations. This method of introduction of salvarsan into the body was abandoned because of the pain and necrosis that followed it. These undesirable sequelæ made it impossible to repeat the injections, and because repeated intravenous injections can be easily given, the intravenous method has almost entirely replaced the intramuscular.

Last year I studied the rate of absorption of arsenic following the intramuscular injection of salvarsan into rabbits; so when neosalvarsan was introduced, it seemed advisable to compare the local action and absorbability of this preparation with that of the older one. The present communication gives the results of this study.

### METHODS.

Approximately 5 per cent. solutions or suspensions of the drug were prepared. With salvarsan three forms were used: (1) alkaline solution prepared by dissolving the drug in water and rendering it alkaline with normal sodium hydrate; (2) neutral suspension after the method devised by Wechsellmann, first rendering the watery solution alkaline with sodium hydrate and then neutralizing it with acetic acid; (3) suspension of the acid salvarsan directly in mineral oil. The neosalvarsan was dissolved directly in sterile, freshly distilled water. One cubic centimeter of the solution or suspension was injected into each muscle.

Full grown rabbits were selected for the experiments. The large

\* Received for publication, October 25, 1912.



erector spinæ muscles in the lumbar region were used for the injection. A large bore needle was inserted directly into the muscle at a point one centimeter above the transverse line connecting the crests of the ilia, and one centimeter from the midline. After the needle was well in the muscle, it was pointed towards the head and advanced another centimeter. After the needle was placed, the syringe was attached and the solution or suspension was injected. Pressure was then made over the site of insertion of the needle during its withdrawal to prevent leakage back along the needle track. Both erector spinæ muscles were used in each rabbit. At the time of the injections duplicates of the various preparations were put aside for determination of arsenic content. The animals were sacrificed at weekly intervals, the muscles removed, and the necrotic area and surrounding muscle analyzed for their arsenic content.

The analyses were made according to the method described by Norton and Koch.<sup>1</sup> The muscles were finely minced, placed in Kjeldahl flasks, and twenty-five cubic centimeters of concentrated sulphuric acid were added. The mixture was slowly heated until the muscle was completely dissolved, then the moist incineration was allowed to proceed at a higher temperature until the solution was clear. This clear solution was transferred to beaker flasks, neutralized with sodium hydrate, then made alkaline with sodium bicarbonate, and titrated with N/20 iodine solution, starch being used as an indicator. The arsenic was thus determined in terms of arsenious acid. With care to prevent drying and subsequent charring of the mixture, this method has been found to be quite accurate.

#### CHANGES IN THE MUSCLE AT THE SITE OF INJECTION.

Necrosis of the muscle occurred uniformly after injection of both salvarsan and neosalvarsan. The gross appearance of the necrotic area was practically the same from alkaline, neutral, or acid salts of salvarsan, and in specimens removed soon after the injections consisted of a brownish or yellowish gray, dry, structureless mass surrounded by a narrow, yellow, soft, granular zone. The muscle adjacent to this was rather pale. The necrotic areas produced by the various injections were of the same size for six weeks, at the

<sup>1</sup> Norton, F. A., and Koch, A. E., *Jour. Am. Chem. Soc.*, 1905, xxvii, 1247.

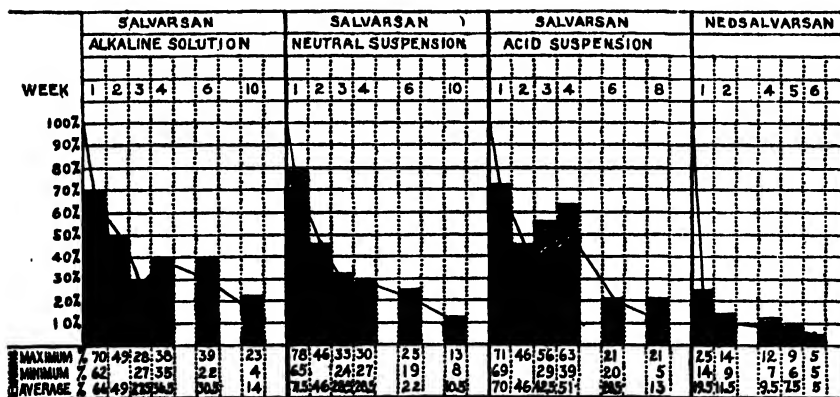
end of which time they were surrounded by a firm connective tissue capsule from which they could be rather easily shelled out. At the end of ten weeks the necrotic areas were about two thirds of their original size and were dry, leathery, surrounded by a thin, yellow zone, and enclosed in a dense, fibrous capsule. Microscopic examination of such a mass (figure 1), removed two weeks after injection of an alkaline solution, showed the central portion to consist of swollen, structureless muscle fibres, in a frail meshwork. Toward the periphery were numerous pale staining polymorphonuclear leucocytes, and passing outward a zone of intense polymorphonuclear infiltration was seen. Many of these cells had undergone fragmentation. Scattered through this zone were a few necrotic muscle fibres. Surrounding the zone of cellular infiltration was a thin zone of newly formed connective tissue, beyond which the muscle was normal with the exception of a few fibres which had a hyaline appearance. A necrotic area resulting from the injection of an oily suspension of the acid salt showed the same microscopic appearance as that produced by the injection of the other salts, except that the zone of newly formed connective tissue was more extensive.

After the injection of neosalvarsan, the area of muscle involved was the same size as that following the injection of salvarsan. Specimens removed soon after the injection showed a grayish yellow necrotic mass in which the muscle fibres could easily be made out, but the granular, limiting zone seen in the necrotic areas following salvarsan injection was not present. At the end of four weeks the mass could be easily shelled out of a fibrous capsule, and after six weeks the necrotic material seemed to be largely absorbed, leaving a brownish yellow leathery mass surrounded by firm, fibrous tissue. Microscopic examination of these areas removed three weeks after injection (figure 2) showed the center to consist of necrotic swollen muscle fibres, surrounded by diffuse, newly formed connective tissue in which were numerous fibroblasts and plasma cells. In the vicinity of the blood vessels were aggregations of small, round cells and plasma cells, with numerous foreign body giant cells. Beyond this zone the muscle fibres were somewhat irregular in outline but the nuclei were normal and the cross striations could readily be distinguished.

The difference in microscopic appearance of the necrotic areas resulting from salvarsan and neosalvarsan injections was quite striking. Following the salvarsan injection the necrosis and polymorphonuclear infiltration were most prominent, while following neosalvarsan injections the inflammation was of a chronic type, and the area was less sharply differentiated from the surrounding tissue.

#### RATE OF ABSORPTION OF THE ARSENIC FROM THE NECROTIC AREAS.

The rate of absorption of arsenic from the necrotic areas is represented graphically in text-figure 1. Two muscles were analyzed each week and the rate of absorption was found to vary somewhat with the different animals. The minimum amount remaining is represented by the heavy shading, the maximum amount by the light shading, and the average by the line. There was a slow steady absorption from all the areas for three weeks after the injection.



TEXT-FIG. 1. The rate of absorption of arsenic from intramuscular injections of salvarsan and neosalvarsan.

After this period the absorption was much slower, so that after ten weeks from 5 to 20 per cent. of the arsenic injected could still be recovered from the muscles. The rate of absorption was most constant after the injection of neutral suspensions, a little more irregular after the injection of the alkaline solution, and most irregular after the injection of the acid suspension. This is what might be expected if the rate of absorption is dependent upon the degree of

injury due to the reaction of the substance injected. Neutral substances would inflict less injury than alkaline or acid salts. Microscopic examination seemed to show that the acid injections resulted in the formation of thicker fibrous capsules, which doubtless prevented rapid absorption.

At the end of ten weeks the necrotic areas were tested for salvarsan by means of the Abelin<sup>2</sup> reaction. The muscles were extracted with N/10 hydrochloric acid, the extract was filtered, and the Abelin test was applied to the filtrate. Only one showed a positive reaction. This was the muscle into which the alkaline solution had been injected and it contained 23 per cent. of the arsenic injected. These results indicate that the arsenic still present in the muscle at this time was in some other form than salvarsan.

The effect of concentration of salvarsan in both alkaline and acid solutions upon the rate of absorption was also studied. In the muscles injected with 0.5, 1, 2, and 3 per cent. solutions, between 20 and 30 per cent. of the arsenic was still present at the end of three weeks, and in the muscles injected with 4 and 5 per cent. solutions, about 40 per cent. of the arsenic remained. The less concentrated solutions were thus shown to be only slightly more readily absorbed than the more concentrated ones.

In striking contrast to the rate of absorption of salvarsan was that following intramuscular injections of neosalvarsan. During the first week as much arsenic was absorbed as after six weeks of salvarsan. After the first week the small amount of arsenic remaining in the muscles was slowly absorbed, about 5 per cent. remaining at the end of six weeks. When the difference in the microscopic picture of the necrosis produced by salvarsan and neosalvarsan is recalled, one is inclined to consider that the intense necrosis produced by the former has much to do with the difficulty in absorption. The solubility of neosalvarsan combined with its neutral reaction probably explains its superiority over the old form for intramuscular injections.

<sup>2</sup> Abelin, J., *München. med. Wchnschr.*, 1911, lviii, 1002.

## SUMMARY.

Intramuscular injection of salvarsan and neosalvarsan in rabbits always produces necrosis of the muscles. A much more intense reaction is produced by salvarsan than by neosalvarsan.

The rate of absorption of arsenic following intramuscular injections of salvarsan is very slow, while following intramuscular injections of neosalvarsan between 75 and 85 per cent. of the arsenic is absorbed during the first week. The subsequent absorption is quite slow.

## EXPLANATION OF PLATE 5.

FIG. 1. Section through the border of the necrotic area following the intramuscular injection of salvarsan.

FIG. 2. Section through the border of the necrotic area following the intramuscular injection of neosalvarsan.



FIG. 1.

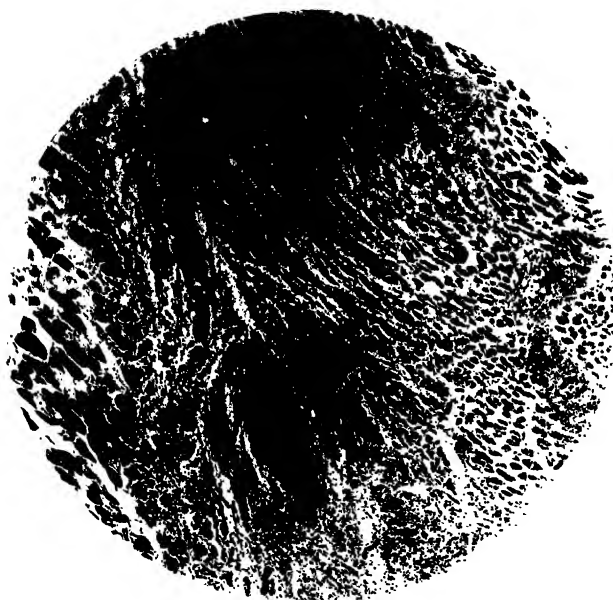


FIG. 2.

(SWIFT: Absorption of Arsenic.)



## INTESTINAL ABSORPTION IN INFANTILISM.\*

By F. H. MCCRUDDEN AND H. L. FALES.

(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)

Previous investigation has shown that in certain cases of infantilism the amount of nitrogen and other elements in the urine is very low, and the amount of these elements in the feces is high. It occurred to us that the losses through the feces might be responsible for the low plane of metabolism and for the failure to develop. To test this possibility, a diet rich in protein was fed for a long period to a dwarf (F. S., reported in earlier papers). Losses of nitrogen through the feces due to inadequacy in the digestion or absorption of protein would, of course, be exaggerated by the high protein diet and would give rise to even higher values for the nitrogen of the feces. At the same time such a diet should raise the plane of metabolism and lead to improvement in the clinical condition.

A high protein diet was maintained for over two months, but attacks of eczema, possibly attributable to the diet, led to its discontinuance, so that not much stress can be laid on the clinical observation that there was no improvement in growth. The chemical examinations were more helpful. Table I shows the results of examinations made before the high protein diet was begun, and table II those made after the diet had been continued for several weeks.

TABLE I.

Date.	Urine.						Nitrogen in feces.
	Total nitro- gen.	Kreatinin.	Kreatin.	Calcium oxide.	Magnesium oxide.	Phosphorus pentoxide.	
Oct. 23, 1911	4.54	0.253	0.107	0.0144	0.223	1.230	1.34
Oct. 24, 1911	2.70	0.238	0.022	0.0192	0.133	0.772	3.32
Oct. 25, 1911	2.66	0.205	0.048	0.0112	0.140	0.780	1.75
Oct. 26, 1911	2.50	0.213	0.034	0.0110	0.130	0.832	1.48

\* Received for publication, November 8, 1912.



TABLE II.

Date.	Urine.						Nitrogen in feces.
	Total nitrogen.	Kreatinin.	Kreatin.	Calcium oxide.	Magnesium oxide.	Phosphorus pentoxide.	
Dec. 14, 1911	12.50	0.386	0.800	0.0156	0.1254	1.633	1.076
Dec. 15, 1911	15.36	0.425	1.015	0.0224	0.1688	1.991	1.225
Dec. 16, 1911	20.80	0.519	1.864	0.0340	0.1830	2.581	1.931
Dec. 17, 1911	12.88	0.337	0.863	0.0216	0.1712	1.822	0.797

In spite of the fivefold increase of food protein in the second period, the nitrogen of the feces is not only not increased, but actually decreased,—a finding indicating that the high nitrogen of the feces on the usual diet can not be due to any inadequacy in the digestion or absorption of protein. The results are in accord with those of previous experiments<sup>1</sup> in indicating that the nitrogen in the feces in infantilism is of the same origin as that in normal feces, being chiefly excretory and not representing unabsorbed food residue.

The increased excretion of nitrogen, phosphate, and, to a less extent, magnesium in the urine on a high protein diet is further evidence that there is no considerable disturbance in the digestion or absorption of protein. It is evident too that we can raise the plane of the metabolism of the elements of the soft tissues without affecting either growth or the metabolism of calcium.

The effect of the high meat diet on the kreatin excretion is important. It will be observed that the amount of kreatin excreted runs parallel day by day with the total nitrogen excretion, so that at least some of the kreatin excreted must have an exogenous origin. Folin<sup>2</sup> has found that kreatin of the food appears again in the urine only when the nutrition of the individual is on a high plane, so that it is fair to assume that the nutrition of our patients is on a high plane. The results seem to exclude the possibility that the failure to develop depends on a condition of malnutrition of the soft tissues or on a fundamental inability to engage in a higher rate of metabolism.

<sup>1</sup> McCrudden, F. H., and Fales, H. L., *Jour. Exper. Med.*, 1913, xvii, 20.

<sup>2</sup> Folin, O., *The Chemistry and Biochemistry of Kreatin and Kreatinin*, Olaf Hammarsten, Festschrift, Upsala, 1906.

The results are in accord with those of our previous work in indicating that there is no disturbance in the digestion, absorption, or utilization of protein in the dwarfs of this type. Large quantities of protein food are digested and absorbed as readily as small quantities without any observable favorable effect on the skeletal abnormalities or on the abnormalities in the calcium metabolism. The general nutrition appears to be good and on a high plane.

## THE CAUSE OF THE FAILURE TO DEVELOP IN INFANTILISM.\*

BY F. H. MCCRUDDEN AND H. L. FALES.

(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)

Changes in metabolism have been found in certain forms of dwarfism.<sup>1</sup> The nitrogen, magnesium, and phosphate of the urine were low, and the calcium of the urine was so low as to be practically negligible. The feces, on the other hand, were large and contained in great excess the various elements which were so low in the urine, the amount of calcium being even greater than that in the food. In other words, the metabolism was found to be on a very low plane, and there was considerable loss of material, especially calcium, through the feces.

One of our patients, F. S., was under observation and treatment for a long period, and some of the clinical features of the disorder showed marked improvement.

In November, 1910, the patient was a puny little child, so weak that he lifted his feet with difficulty in walking. His height was 96 cm., and his weight 13.6 kilos. He had very poor muscular control and when lying on his back was unable to lift his trunk to the sitting position by contraction of the abdominal muscles. The stools were large and in odor very offensive, and only the smallest quantities of food could be given without causing increased foulness.

By May, 1911, there was a striking change. He had become a fat, rosy, cheerful, healthy boy, could run, and had excellent muscular control. His weight had increased to 20 kilos, but his growth had been slight, an increase of about 1 cm. in height during the winter.

Briefly stated, there appeared to be a striking improvement in the general health and nutrition of the soft tissues, but the abnormalities referable to the skeletal system did not improve. The bones remained small and fragile and the patient did not grow.

In view of the number of changes in metabolism which had previously been found, it seemed worth while to carry out in June, 1911,

\* Received for publication, November 8, 1912.

<sup>1</sup> McCrudden, F. H., and Fales, H. L., *Jour. Exper. Med.*, 1912, xv, 450.

at a period when the general health had so much improved, a complete balance metabolism experiment similar to that carried out in December, 1910, with the hope of finding which of the metabolic disturbances were fundamental and associated with the failure of the skeletal system to develop.

Table II shows the results of the observations. The analytical and other technical details of the observations have already been described. The results of the previous balance experiment are given in table I for comparison.

TABLE I.

Day.	Total weight of dried feces.	Nitrogen.	Calcium oxide.	Magnesium oxide.	Phosphorus pentoxide.
Urine { 1 2 3 4 5 6		2.75	0.010	0.005	0.512
		2.76	0.016	0.070	0.520
		3.02	0.021	0.086	0.572
		2.75	0.012	0.074	0.497
		2.51	0.009	0.054	0.398
		2.32	0.040	0.111	0.211
Total.....		16.11	0.108	0.400	2.710
Feces { 1 2 3 4 5 6	29.60	1.707	1.965	0.213	0.855
	14.32	0.868	0.750	0.009	0.767
	58.50	3.532	2.744	0.579	2.495
	40.35	2.840	1.733	0.339	1.077
	52.55	2.286	1.618	0.331	1.072
	35.75	2.288	1.451	0.229	1.073
Total.....	231.1	13.52	10.26	1.700	7.34
Total amount excreted .		29.63	10.37	2.10	10.25
Total in food.....		35.64	6.35	2.43	11.91
Retention.....		6.01	—	0.33	1.66
Loss.....		—	4.02	—	—

It will be noted that the quantities of nitrogen, phosphate, and magnesium in the urine have increased considerably, and the phosphate and magnesium of the feces have decreased as the patient has improved. The large stools containing excessive quantities of nitrogen and calcium still persist, and the calcium in the urine remains low.

We have succeeded, then, in improving the general state of nutrition of the patient without, however, improving the disturbance of growth in the skeletal system or correcting the failure to develop. This clinical fact would seem to indicate that the failure to develop

does not depend upon a general nutritional disturbance, but upon some specific disturbance referable to the skeletal system. With the improvement in general nutrition, chemical observations show that the metabolism of those elements associated with the soft tissue has risen to a higher plane. The metabolism in calcium, on the other hand, an element important for bone growth, remains disturbed.

TABLE II.

Day.	Total weight of dried feces.	Nitrogen.	Calcium oxide.	Magnesium oxide.	Phosphorus pentoxide.	Sulphur.
After improvement {	1	5.33	0.016	0.210	1.600	0.312
	2	4.26	0.019	0.231	1.268	0.236
	3	5.16	0.026	0.242	1.287	0.317
	4	5.39	0.022	0.220	1.396	0.348
Urine... {	5	4.58	0.016	0.218	1.308	0.345
	6	5.84	0.019	0.226	1.495	0.403
	7	5.63	0.018	0.206	1.434	0.452
	8	5.51	0.019	0.257	1.474	0.356
	9	6.04	0.014	0.146	1.285	0.407
	10	7.11	0.029	0.230	1.483	0.467
Total .....		54.85	0.198	2.186	14.03	3.643
Feces... {	1	60.91	3.46	1.218	0.237	0.735
	2	31.83	1.77	0.548	0.096	0.325
	3	61.91	3.62	1.245	0.225	0.691
	4	38.60	2.44	0.742	0.109	0.498
	5	44.77	2.61	0.910	0.107	0.563
	6	65.40	3.61	1.320	0.256	1.090
	7	35.97	2.10	0.653	0.100	0.436
	8	49.32	2.77	0.907	0.170	0.523
	9	48.53	2.97	0.976	0.192	0.602
	10	35.88	1.97	0.936	0.105	0.586
Total .....	473.12	27.32	9.455	1.659	6.049	2.473
Total amount excreted.		82.2	9.65	3.84	20.08	6.12
Total in food .....		110.5	16.85	5.26	31.55	6.55
Retention .....		28.3	7.20	1.42	11.47	0.43

Weight at beginning of experiment, 19.0 kilos; weight at end, 20.2 kilos.

The excessively low calcium of the urine must mean one of three things: (1) either the kidneys are not doing their work properly; (2) the calcium content of the blood is very low; or (3) the calcium of the blood exists in some combination which does not permit of its excretion through the kidneys. There is, however, no other evidence of improper functioning of the kidney, so that one of the

other two conditions must be responsible for the trouble; and in the excessive loss of calcium through the feces, we have a plausible explanation for a low calcium content of the blood.

The results indicate that the general retardation in growth is secondary to a failure to develop on the part of the skeletal system. And the disturbance of calcium metabolism, associated with the frail, thin bones, strongly suggests that the bones have not sufficient calcium in proper form at their disposal. As pointed out by Rubner,<sup>2</sup> there may be two causes for failure to grow: first, a lack of material for growth; and second, an absence of the tendency to grow,—this being the tendency normally seen in the young, but absent in the adult. In the form of underdevelopment described in this paper, there appears to be some disturbance in the supply of material for growth, rather than in the tendency to grow.

<sup>2</sup> Rubner, M., *Arch. f. Hyg.*, 1908, lxvi, 1.

## THE INFLUENCE OF THE VAGUS NERVES ON THE FARADIZED AURICLES IN THE DOG'S HEART.\*

By G. CANBY ROBINSON, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research, New York.)

PLATES 71 TO 78.

The experiments on which this paper is based were undertaken in order to study the influence of the vagus nerves on auricular fibrillation. The problem was suggested by the study of a patient suffering from transient attacks of auricular fibrillation, whose heart was affected to an unusual degree by pressure over either of the vagi. The patient was of neurotic temperament, and the circumstances under which the attacks of auricular fibrillation occurred indicated that the controlling nervous mechanism of the heart might have played an important part in determining the onset and determination of the attacks. The case itself will be described in detail in another communication. The experiments were undertaken with the idea of determining whether the activity of the inhibitory mechanism played any part in rendering the heart susceptible to auricular fibrillation or in preventing its occurrence.

Certain experimental investigations have been carried on regarding the influence of the vagi on the fibrillating auricles. There has not been, however, entire agreement of interpretation of results by the various workers. Some have said that vagus stimulation may cause auricular fibrillation or at least increase the susceptibility of the heart to such a condition, while others consider that it prevents auricular fibrillation or causes established fibrillation to cease.

Knoll (1), Hewlett (2), Winterberg (3, 4, 5), and Cushny (6) point out that vagus stimulation increases the susceptibility of the heart to auricular fibrillation, and that faradization of the nerves alone may sometimes produce the condition in the mammalian heart. Winterberg (4, 5), who has investigated this question more thoroughly than anyone else, states that a weaker faradizing current is required to throw the auricles into fibrillation when combined with vagus stimu-

\* Received for publication, January 20, 1913.

lation or when drugs which raise the vagus tone have been administered. than when the auricles alone are stimulated. He also says that vagus stimulation may fix an auricular fibrillation set up by a short period of faradization, so that it continues after auricular faradization is removed.

McWilliams (7), Fischel (8), Philips (9), Hirschfelder (10), Garrey (11), Lewis (12), and Cushny (6) state that vagus stimulation, on the other hand, arrests auricular fibrillation. Winterberg (4) has noted that stimulation of the vagi may both make the heart more susceptible to auricular fibrillation and also arrest it when established. McWilliams, Lewis, Winterberg, Rothberger and Winterberg (13), and Hewlett describe the effects which vagus stimulation has on established auricular fibrillation, and all agree that it causes the fibrillary movements to become more rapid, finer, and less easily seen. Rothberger and Winterberg consider this effect to be the same as that which causes a great diminution or disappearance of the P wave in the electrocardiogram of the normal heart-beat. Finally, Kronecker and Spillata (14) conclude that vagus stimulation does not affect auricular fibrillation.

#### METHODS.

The material upon which the present communication is based consists of twenty-three experiments on dogs. Medium sized or rather large animals, weighing from 6,800 to 17,000 grams, were used. As the experiments were varied, the number bearing on the various points considered is not always the same, and so will be mentioned in each instance. Records were obtained by means of the string galvanometer (Edelmann), and in one experiment by suspension curves from auricles and ventricles as well. Direct observation of the heart also proved useful.

The usual method of experimentation was as follows: The animal was etherized by intratracheal insufflation (Meltzer-Auer method), and German silver electrodes over cotton soaked with salt solution were applied to the right fore leg and the left hind leg. The string of the galvanometer was always adjusted so that one millivolt caused a movement of ten millimeters on the record. After an electrocardiogram had been taken, the chest was opened by means of a lateral incision through the third or fourth intercostal space. The right auricle was exposed by a small incision of the pericardium, and to it were attached, about five millimeters apart, two small clips wrapped separately in rubber tissue. From these clips light wires ran to the secondary coil of a Harvard inductorium. Both vagi were then dissected out in the neck, but not, as a rule, cut. The



right auricle was usually stimulated for fifteen seconds by a faradic current. If the auricles did not continue to beat tumultuously after the faradization ceased, it was repeated three times at intervals of one minute. If still unsuccessful, the vagi were cut and stimulation of the auricle was repeated in the same manner. Several minutes were allowed to elapse between the two sets of stimulations. If rapid, tumultuous auricular activity became established during the first set of stimulations, electrocardiograms were made before and after cutting the vagi, so that the effect of cutting the nerves could be observed. After cutting the vagi, the peripheral end of each nerve was laid in a shield electrode, which was protected by rubber tissue and dry gauze. The effect of stimulation of both the right and left vagi was investigated when the auricles were affected by faradization, as well as when the heart was beating normally. When the altered activity following auricular faradization could not be established so that it continued independently, vagus stimulation was begun immediately before ending auricular faradization. As the altered auricular activity almost always peristed during vagus stimulation, the effects of stimulating the nerves upon it could be studied in this way.

The strength of the faradizing current did not bear a constant relation to the length of time the auricles remained affected after faradization was ended. A current just strong enough to keep the auricles constantly in a state of tumultuous activity seemed as effectual as stronger currents in causing this activity to continue after faradization had ceased. With one two-volt cell, it was found in several experiments that when the secondary coil of the inductorium was out eighty or ninety millimeters, the current was just strong enough to maintain auricular fibrillation. To be well within this limit but to avoid very strong currents, the auricle was usually stimulated with the secondary coil sixty millimeters out. The vagi were stimulated by a faradic current from an inductorium, the secondary coil of which was extended fifty millimeters. The strength of this current is the same as that used by Cohn (15) in his experiments on the vagus control of the normally beating heart, so that the results of his experiments and those of this series are comparable.

The importance of considering the effect of stimulating each vagus separately has become obvious since Cohn (15) has shown that there is a definite difference in function between the two nerves almost constantly demonstrable in dogs. This difference is as follows: when the right vagus in the dog is stimulated, there is, in a large proportion of cases, stoppage of the entire heart, while when the left is stimulated, the auricles beat at a rate not greatly reduced while partial heart-block or ventricular asystole usually occurs. The effect of stimulation of the two vagi must therefore be considered separately.

#### RESULTS.

1. *The Effect of Faradization on Auricular Activity.*—The auricles always beat in a rapid tumultuous manner during faradization. In some dogs this altered rhythm ceased synchronously with the end of faradization if no other measures were employed, while in others it continued and became established. This altered rhythm became established by faradization alone in eleven experiments, while in four others its establishment was accomplished by combining auricular faradization with vagus stimulation. It continued in these fifteen experiments from five minutes to over an hour after faradization, thus giving opportunities for studying its character and the effect of vagus stimulation upon it.

Direct observation of the exposed heart showed that the auricular activity following faradization consisted in very rapid movements, apparently contractions of the whole auricles, which were sufficient to produce definite movements of the recording tambour attached to the auricular myocardiograph (figure 11). Besides this rapid auricular tachycardia, fine fibrillatory movements in the various fibers could be seen. During the right vagus stimulation, the effect of which will be discussed later, the coarser movements ceased and the typical fine fibrillations persisted, and when the stimulation was removed the coarser movements could be seen definitely gradually returning and being coexistent with the fine fibrillations. This direct observation was confirmed by my colleague, Dr. Cohn, who first observed this phenomenon in one of my experiments.

The electrocardiogram (figure 7) shows no waves which resemble those caused by the normal auricular contractions, but between the

rapidly and irregularly recurring ventricular complexes, irregular waves of various sizes occur usually at a rate averaging 500 to 600 per minute. The waves may be four to five millimeters in height, but the larger waves are often interrupted by much smaller, finer, and more rapid movements of the string. The irregularities in the size and shape of the ventricular complexes are probably caused by various degrees of coincidence with the waves of auricular activity.

It is evident that the altered activity of the auricles usually resulting from faradization is not true fibrillation alone, but consists of two different forms of cardiac activity occurring simultaneously in the auricles; namely, tachycardia and fibrillation.

The auricular activity following faradization has been described and recorded by electrocardiograms by Rothberger and Winterberg and by Lewis. The records obtained by these observers agree closely with ours. From their direct observations they describe different grades of fibrillation, varying in extent of auricular movements. Lewis states that actual correspondence between the auricular activity and the electrocardiograms may be found only rarely, while Rothberger and Winterberg consider that no correlation can be made out. Beside these various grades of fibrillation Rothberger and Winterberg describe finally what they call *unreines Schlagen*. This form of auricular activity consisted of definite contractions of the auricles which affected at least the main mass of the musculature, together with either fibrillatory movements seen more or less distinctly, especially on the margins of the auricles, or weak peristaltic waves running across the auricles, occurring at the same time. This peculiar combination of the normal beat and delirium caused no corresponding impression on the electrocardiogram. The auricular wave was usually somewhat split, and here and there the rhythm was disturbed. They do not state how frequently this type of auricular activity was seen or under what conditions it occurred, and no further comment is made upon it. It is evident from their description that Rothberger and Winterberg have observed the same form of auricular activity which resulted almost constantly from auricular faradization in our experiments. The various grades of fibrillation which these observers and Lewis describe did not appear in our experiments except under conditions to be discussed,

although a pure auricular tachycardia, as observed by Hirschfelder and others, followed faradization sometimes in some of our experiments. The rapid auricular activity may be conveniently designated tachycardia in this paper, as this was practically always the most obvious effect of auricular faradization.

2. *The Influence of Cutting Both Vagi.* (a) *On the Susceptibility of the Normally Beating Heart to Auricular Faradization (Fourteen Experiments).*—Prolonged auricular tachycardia was more readily produced by faradization after cutting both vagi in three experiments, while in ten no change in the susceptibility of the auricles to faradization could be detected. In one tachycardia seemed more readily produced before than after cutting the nerves. The protocol of experiment 12 shows the positive influence that cutting the vagi may have.

11:20 A. M.	Auricular faradization, 15 seconds.	Tachycardia for 10 seconds after end of faradization.
11:21 A. M.	Auricular faradization, 15 seconds.	Tachycardia for 3 seconds after end of faradization.
11:22 A. M.	Auricular faradization, 15 seconds.	Tachycardia for 8 seconds after end of faradization.
11:23 A. M.	Right vagus tied and cut.	
11:24 A. M.	Left vagus tied and cut.	
11:27 A. M.	Auricular faradization, 15 seconds.	Tachycardia for 19 minutes after end of faradization. Normal beat returned only after four vagus stimulations.

(b) *On the Heart with Established Auricular Tachycardia (Eight Experiments).*—In these experiments prolonged tachycardia was established with the vagi intact, so that records could be obtained before and after they were cut. This procedure had apparently very little effect on the auricular activity. In six experiments no effect could be detected in the electrocardiograms, while in one the waves of auricular activity were considerably slowed and in another rendered larger and more distinct. The ventricular rate was increased in four experiments, while in three there was practically no change. In the one other experiment, no conclusions can be drawn, as other procedures may have influenced the ventricular rate. In several experiments there was seen a gradual increase in ventricular rate

as auricular tachycardia continued, so caution is necessary in interpreting these results, but in three, at least, of the four positive experiments, the results seem quite definite. This is seen especially well in experiment 13 where the ventricular rates during auricular tachycardia were as follows:

86 per minute	1 minute after onset.
160 per minute	5 minutes after onset.
168 per minute	7 minutes after onset (figure 1).
Both vagi cut	9 minutes after onset.
300 per minute	12 minutes after onset (figure 2).
240 per minute	20 minutes after onset.

When the heart was beating normally, cutting the vagi caused a definite increase in the rate of the heart-beat in thirteen of seventeen experiments.

3. *The Influence of Stimulation of Each Vagus Nerve.* (a) *On the Auricular Activity.*—Stimulation of the right vagus nerve (nineteen experiments) had a definite and almost constant effect on the auricular tachycardia, which could be observed by watching the heart directly and by changes in the electrocardiograms. Suspension curves were made in one experiment in which the heart behaved in a manner entirely typical of the other experiments. The auricular activity could be seen to undergo the marked change mentioned above. Instead of the auricles moving about rapidly as a whole, they became practically stationary, allowing the true fibrillatory contractions of the various muscles fibers to be plainly seen. They were similar to those observed in the ventricles when in fibrillation. In the electrocardiograms the undulations representing auricular activity became more rapid, blurred, and often almost disappeared. The large waves, sometimes four to five millimeters in height, rarely appeared (figures 5, 8, 12). In the experiment in which suspension curves were also made of the right auricle and right ventricle, it was found that the auricular tachycardia produced movements of the recording tambour nearly as distinct as those produced by the normally beating heart (figures 10 and 11). When the right vagus was stimulated (figure 12), the waves of the auricular suspension curve became smaller and smaller and finally disappeared, synchronously with the replacement in the electrocardiogram of very fine

small waves of true fibrillation for the well defined coarse waves of auricular tachycardia. Finally, it may be mentioned again that soon after right vagus stimulation, distinct contractions of the entire auricles could be seen accompanying but not replacing the fine fibrillatory movements in the auricular walls. It is evident, therefore, that when the right vagus was stimulated, it exerted its inhibitory influence on the auricular tachycardia, causing it to cease, while the true fibrillatory movements were apparently unaffected. This effect of right vagus stimulation occurred in seventeen of the nineteen experiments (89.5 per cent.), and may therefore be considered characteristic. It is apparently similar to that which has been described by other experimenters already mentioned. In one of the two instances where the typical effect was not observed, the character of the record changed in an unusual manner. At first the waves were blurred and very irregular. Then separate, distinct little waves, resembling the normal P waves, appeared at a rate of 560 per minute, and continued to occur as long as the vagus was stimulated. Here the auricular tachycardia apparently succeeded in reasserting itself. In the second instance no change could be observed in the auricular activity. In this experiment even the normally beating heart did not react to right vagus stimulation in the usual manner, as a distinct delay occurred between the time of onset of stimulation and stoppage of the heart.

The effect of left vagus stimulation on auricular fibrillation was studied in seventeen of the nineteen experiments in which right vagus effects were observed. A constant difference was observed between the influence of right and of left vagus stimulation in twelve of these seventeen experiments (70.6 per cent.). During left vagus stimulation direct inspection showed that the coarser movements of the auricles were not disturbed, but appeared sometimes perhaps even more distinctly. It was difficult to determine with certainty whether the fibrillatory movements ceased or were influenced. In some of the electrocardiograms there was seen slight blurring and quickening in rate of the waves of auricular activity similar to, but always less than, that seen with right vagus stimulation in the same animal. In other records the waves of auricular activity occurred more regularly, uniformly, and distinctly during

left vagus stimulation than before, and only the larger waves appeared, the small, fine rapid movements of the string which interrupted the larger waves practically disappearing (figures 6, 9, and 13). While some records, those first mentioned, indicate that the difference between the effects of right and left vagus stimulation seems to be one of degree rather than of kind, the others show that the type of response to the two nerves seems different. The distinctness and regularity with which the waves of auricular activity appeared may have been due to the removal of the confusing ventricular complexes, but the records suggest strongly that left vagus stimulation affected the auricular activity in an opposite manner from right vagus stimulation, and a change was produced which, instead of blurring and quickening the auricular waves, rendered them clearer, more uniform, and more regular. '

The suspension curve from the auricles (figure 13) shows that the coarser auricular activity was not disturbed during left vagus stimulation, while during right vagus stimulation the suspension curve gave no evidence of motion. It seems evident, therefore, that stimulation of the left vagus often had practically no influence on the auricular tachycardia, while there is some evidence that it exerted an inhibitory influence on the true fibrillation.

In four experiments no definite difference could be made out between the effect of the two vagi on the auricular activity resulting from faradization. In three of these fairly marked changes occurred when each nerve was stimulated, while in the fourth the effect of each nerve was very slight.

The effect of stimulation of each nerve on the normally beating heart was studied in seventeen experiments, and in twelve of these (70.6 per cent.) the characteristic differences between the effects of stimulation of the two nerves were seen, just as described by Cohn (figures 3 and 4). Although the difference in effect of stimulation of the two nerves on the normally beating heart is more striking, it does not appear to occur more constantly than the difference in effect on auricular activity after faradization.

The difference in the effects of stimulation of the two vagi are seen in the figures. The curves in figures 3 to 6 are from one experiment (No. 18) and show the action of the two vagi on the heart,

both when it was beating normally and when auricular tachycardia was present. In this experiment prolonged auricular tachycardia could not be established, so vagus stimulation was begun before the end of auricular faradization. The curve in figure 7 (experiment 11) is an electrocardiogram obtained after the auricles had been faradized, and figures 8 and 9 from the same experiment show the changes in the electrocardiograms which resulted from vagus stimulations. Figures 10 to 13 show suspension curves from the auricles and ventricles, and illustrate the changes in auricular activity which have been described.

The results of the various experiments indicate that the length of time that tachycardia had been established did not apparently influence the vagus action upon it, and that the difference in effect of stimulation of the two nerves was observed regardless of which nerve was stimulated first.

(b) *On Reestablishment of the Normal Sequential Beat (Thirteen Experiments).*—It was frequently seen that after auricular tachycardia had become established and had continued for several minutes without showing any signs of ceasing spontaneously, it would cease several seconds after the end of stimulation of one of the vagi (figure 14). It was concluded from the constant behavior of the dog's heart in this respect that vagus stimulation was the cause of the reestablishment of the normal sequential beat. Of thirteen experiments, stimulation of each nerve was followed by the reestablishment of the normal beat in seven. Left vagus stimulation alone produced this result in three, right vagus stimulation produced it in one, while in two experiments the normal beat never returned as a result of vagus stimulation. In other words, right vagus stimulation caused cessation of the abnormal auricular activity in 61.5 per cent. of the experiments, while left vagus stimulation had this effect in 76.9 per cent. Although numerically considered, this difference in the two nerves is not great, it is clear that stimulation of the left vagus was more effectual in reestablishing the normal sequential beat than that of the right. Vagus stimulation did not usually seem to produce any permanent change in the susceptibility of the auricles to faradization, although in two or three experiments the auricles apparently became more resistant to faradization after the vagi had been stimulated a number of times.



(c) *On the Susceptibility of the Auricles to Faradization.*—In the experiments in which prolonged auricular tachycardia could not be established by auricular faradization alone, the influence of vagus stimulation in aiding the auricles to maintain prolonged tachycardia could be observed. The method of determining this influence was to throw the auricles into tachycardia by faradization for fifteen seconds, and to begin vagus stimulation just before ending auricular faradization. Vagus stimulation was continued for five to eight seconds. Twice in ten experiments right vagus stimulation caused the tachycardia to become established and to continue for some minutes after the vagus stimulation ceased. The same result was obtained in two other experiments with left vagus stimulation. In seven of the eight experiments in which faradization combined with right vagus stimulation did not succeed in establishing the tachycardia, the auricles always remained in a state of tachycardia as long as the nerve was stimulated. It seemed quite evident that the auricular tachycardia was maintained by vagus action. In the remaining experiment tachycardia continued during three out of four vagus stimulations, so it can be said that when the auricles were thrown into a state of tachycardia by auricular faradization, they continued almost invariably in such a state as long as the right vagus was stimulated. With left vagus stimulation this result was obtained in but five of eight experiments, and in three experiments in which right vagus stimulation maintained auricular tachycardia, left vagus stimulation, although active in other respects, failed to do so. It appears that stimulation of the right vagus nerve was more effectual than stimulation of the left in aiding the auricles to maintain the activity set up by faradization, a fact that was especially noted in the protocols of several experiments.

(d) *On Initiating Auricular Tachycardia.*—In two experiments on hearts in which auricular tachycardia was readily established by faradization, it was also initiated by right vagus stimulation alone. In one (No. 25) it began on two occasions during vagus stimulation (figure 15), and in the other (No. 13) this occurred two seconds after the stimulation ceased (figure 16). This first experiment (No. 25) is especially interesting because auricular tachycardia was stopped as well as started by right vagus stimulation. Twenty-

four minutes after the tachycardia had been established by right vagus stimulation alone (figure 15), it ceased four seconds after stimulation of the same nerve (figure 14). This result was obtained twice in this experiment with right vagus stimulation, but stimulation of the left nerve was also followed by a return of the normal beat two minutes after auricular tachycardia had been set up by right vagus stimulation alone.

Stimulation of the left nerve alone never caused auricular tachycardia in these experiments, although it did follow left vagus stimulation in one case in a long series of vagus stimulations by Dr. Cohn.

The influence of vagus stimulation on the ventricles during the type of cardiac activity which results from auricular faradization will not be considered in this paper.

#### DISCUSSION.

The auricular activity which follows faradization of the auricles is apparently the result of a change in the physiological properties of the auricular musculature. This change seems to alter the excitability and the power of stimulus formation in the auricles and finds expression in the establishment synchronously of auricular tachycardia and true fibrillation. This altered auricular activity persists after faradization of the auricles has been withdrawn, indicating that the altered cardiac properties do not immediately return to their normal state as soon as the exciting cause is removed.

The normal tonic vagus activity has in some animals a definite control over these cardiac properties under the conditions of our experiments, as cutting the vagi sometimes increases the susceptibility of the auricles to faradization. After the auricles take on the activity resulting from faradization, the tonic vagus action on the auricles seems very slight, as but little if any effect can be detected when the vagi are cut during auricular tachycardia. The influence of this procedure on the ventricular activity is, however, in some experiments quite definite, and results in a marked increase in ventricular rate. The result depends obviously upon a change in conductivity in the auriculoventricular conducting system. The depression of auriculoventricular conduction is one of the most obvious

activities of the vagi, and when this depression is removed, the ventricles are stimulated to contraction by a larger number of impulses from the rapidly contracting auricles than when the vagi are intact. That this increase in ventricular rate is not constant when the vagi are cut indicates that conductivity is not always depressed by tonic vagus action.

The effects of vagus stimulation on the auricular activity resulting from faradization are in accord with the effects which stimulation of the two nerves has on the normally beating heart. Stimulation of the right vagus stops the auricular tachycardia just as it stops the auricles in the normally beating heart, and stimulation of the left nerve fails to do so in both instances. True auricular fibrillation obviously is not controlled by right vagus stimulation, while there is insufficient evidence to allow any definite statement as to the effect of left vagus stimulation upon it. The electrocardiograms seem to show, however, that auricular fibrillation may be inhibited by left vagus stimulation while the tachycardia proceeds undisturbed.

Auricular tachycardia in man may sometimes be controlled by pressure over the right vagus nerve, as will be shown by electrocardiograms to be published shortly by my colleagues, Drs. Cohn and Fraser, while Draper and I (16) have published electrocardiograms which indicate that right vagus pressure does not influence auricular fibrillation in man.

The mechanism by which the normal sequential beat is restored a few seconds after vagus stimulation is difficult to understand. Left vagus stimulation seems somewhat more effectual in producing this result than right vagus stimulation, so that it cannot come from inhibitory influences of the auricular tachycardia. More facts are needed before an explanation of this phenomenon can be attempted.

Vagus stimulation increases the susceptibility of the auricles to faradization. This is indicated by the fact that in animals whose auricles are resistant to faradization, the abnormal auricular activity continues after faradization as long as one of the vagi is stimulated. This effect probably results from the inhibitory influence of vagus stimulation on the normal auricular activity. As long as the normal pace-maker is depressed, there is probably a diminished tendency for the abnormal auricular activity to be superseded by the normal

sequential beat. The more marked influence which the right vagus has on the normal auricular activity explains why it is more effectual in holding the auricles in the abnormal activity than the left nerve. It may be that it is through this same mechanism that the abnormal auricular activity can be originated by vagus stimulation alone. If the auricles are in a state very favorable to the establishment of tachycardia and fibrillation, inhibition of the normal pace-maker alone may suffice for the establishment of the abnormal auricular activity. The fact, however, that the tachycardia may come on during right vagus stimulation without showing the usual effect of the stimulation (figure 15), or that it may come on at a time after the stimulation when the normal pace-maker usually reasserts itself (figure 16), does not lend support to this possibility. It is not clear also why a short vagus stimulation following the faradization should assist in the establishment of the abnormal activity of the faradized auricle as an independent auricular activity.

#### SUMMARY AND CONCLUSIONS.

An abnormal auricular activity is produced by faradization of the right auricle of the dog, which frequently becomes established and continues for varying periods of time after faradization is discontinued. This auricular activity consists of a rapid auricular tachycardia coexisting with true auricular fibrillation. In some dogs the auricles are thrown into this abnormal activity more readily by faradization after the vagi have been cut than before. Cutting the nerves has little or no effect on the abnormal auricular activity, but the ventricular rate may be much increased if the vagi are cut after the abnormal auricular activity has been established, apparently because of an improvement in the auriculoventricular conductivity.

Stimulation of the right vagus nerve changes the character of the activity of the faradized auricles by inhibiting the auricular tachycardia while the fibrillation is uninfluenced. Stimulation of the left vagus nerve has little or no apparent inhibitory effect on the auricular tachycardia, but has possibly an inhibitory effect on the auricular fibrillation.

Vagus stimulation increases the susceptibility of the auricles to faradization. The abnormal activity set up by faradization may be

established in hearts otherwise refractory by vagus stimulation of short duration following the faradization. Vagus stimulation usually holds the auricles in the abnormal activity set up by faradization as long as it is continued in hearts in which, without vagus stimulation, the sequential beat always returns as soon as faradization is stopped. The right vagus is more effectual in this respect than the left. In some hearts vagus stimulation alone is capable of initiating the same abnormal auricular activity which is caused by auricular faradization. The normal sequential beat is often restored by vagus stimulation. It replaces the abnormal auricular activity not during, but a few seconds after, the termination of vagus stimulation. Left vagus stimulation is somewhat more effectual in producing this result than right vagus stimulation.

## BIBLIOGRAPHY.

1. Knoll, P., *Arch. f. d. ges. Physiol.*, 1897, lxvii, 587.
2. Hewlett, A. W., *Heart*, 1910-11, ii, 107.
3. Winterberg, *Arch. f. d. ges. Physiol.*, 1908, cxxii, 361.
4. Winterberg, *Arch. f. d. ges. Physiol.*, 1907, cxvii, 223.
5. Winterberg, H., *Ztschr. f. exper. Path. u. Therap.*, 1907, iv, 636.
6. Cushny, A. R., *Am. Jour. Med. Sc.*, 1911, cxli, 826.
7. McWilliams, J. A., *Jour. Physiol.*, 1887, viii, 296.
8. Fischel, R., *Arch. f. exper. Path. u. Pharmacol.*, 1897, xxxviii, 228.
9. Philips, F., *Arch. internat. de physiol.*, 1904-05, ii, 271.
10. Hirschfelder, A. D., *Bull. Johns Hopkins Hosp.*, 1908, xix, 322.
11. Garrey, W. E., *Am. Jour. Physiol.*, 1908, xxi, 283.
12. Lewis, T., *Heart*, 1909-10, i, 306.
13. Rothberger and Winterberg, *Arch. f. d. ges. Physiol.*, 1910, cxxxi, 387.
14. Kronecker, H., and Spillata, F., *Arch. internat. de physiol.*, 1904-05, ii, 223.
15. Cohn, A. E., *Jour. Exper. Med.*, 1912, xvi, 732.
16. Robinson, G. C., and Draper, G., *Jour. Exper. Med.*, 1911, xiv, 217.





## EXPERIMENTS ON THE TRANSMISSION OF SCARLET FEVER TO THE LOWER MONKEYS.\*

BY GEORGE DRAPER, M.D., AND JOHN M. HANFORD, M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research, New York.)*

The subject of the experimental production of scarlet fever in the monkey is at present under discussion. Indeed, it was partly because of the discordant statements in the literature that we were led to undertake a detailed study of the question, as the successful transmission to the lower animals of an otherwise refractory disease has often been followed by an important increase in the fundamental knowledge of the affection. The etiology of scarlet fever is still unestablished and there exists as yet no specific mode of treatment. Furthermore, it is uncertain whether a streptococcus bears a direct or only an indirect relation to the disease, although it is known to be active in causing secondary or concomitant infections, either septicemic or suppurative in nature.

Whatever the causative agent may be, there is no doubt of the inoculability of scarlet fever upon healthy human beings. Proof of this is found in the history of obstetrical practice of a generation ago and in the epidemical nature of the disease. The important and often fatal part played by the streptococcus in the former instance is well known. Stickler's observations tend, moreover, to show that the germ of scarlet fever is able to resist 0.6 per cent. carbolic acid, but the writer fails to state for how long a period. Stickler also supports the view, now generally accepted, that the virus is contained in the secretions of the mucous membrane of the nose and throat.

The results with animals are less convincing. The ordinary laboratory animals appear to be wholly refractory to inoculation with materials carrying the virus. There is general agreement re-

\* Received for publication, February 12, 1913.



garding this point. The question turns upon the susceptibility to inoculation of monkeys, both higher and lower.

In 1904 Grünbaum produced doubtful symptoms in a chimpanzee which he clad in the nightshirt of a scarlet fever patient. In addition to this, the animal's throat was thoroughly rubbed with swabbings from the pharynx of a case of scarlet fever. In 1911 Hektoen and Weaver failed to obtain any result from feeding *Macacus rhesus* monkeys upon milk contaminated with swabbings from the throats of scarlet fever patients. The present revival of interest dates from Cantacuzène's announcement that infection of lower monkeys (*Macacus rhesus*, *Macacus sinensis*, *Cercopithecus cephus*, *Cercopithecus griseoviridis*) was accomplished by inoculating them with blood, pericardial effusion, and emulsion of lymph nodes taken from cases of scarlet fever. Bernhardt soon followed with an account of the successful subcutaneous inoculation of a *Macacus rhesus* with scrapings from the tongue, swabbings from the throat, and serum from a bleb of scarlet fever patients, and also from merely swabbing the throat with infected scrapings from the tongue. In a second report he states that a Berkefeld filtrate prepared from lymph glands produced identical symptoms on injection into *Macacus rhesus* monkeys. From this he concludes that the virus is filterable. We shall return to a consideration of the symptoms described by these authors as evidence of successful infection. The most recent publication on the subject is that of Landsteiner, Levaditi, and Prasek who report the appearance of suggestive but unconvincing symptoms as the result of inoculating a chimpanzee. They proceeded as follows: Three chimpanzees were used. The first animal's throat was rubbed well with a deposit from the tonsils of a child with scarlet fever. Three days later the animal received subcutaneously 75 c.c. of defibrinated blood from another case. Two days later the symptoms began to appear. Death occurred in ten days. The second chimpanzee was inoculated repeatedly as follows:

June 18. 20 c.c. of defibrinated blood, subcutaneously; tonsils swabbed with the deposit from the throat of the patient.

June 19. 10 c.c. of defibrinated blood, subcutaneously; throat swabbing repeated.

June 21. 5 c.c. of emulsion of cervical glands from a fatal case, subcutaneously (material glycerinated).

June 23. 5 c.c. of blood, subcutaneously; tonsils swabbed. Death in six days.

The third chimpanzee was inoculated less intensely with tonsillar deposits and blood, in the throat, subcutaneously, and intraperitoneally. The animal recovered in six days, after a course with angina, false membrane, rise of temperature, and diarrhea. At autopsy a streptococcus was isolated in pure culture from the blood of the two fatal cases. A subsequent protocol indicates that one attack, such as that described for chimpanzee 3, does not confer immunity.

The symptoms upon which all the observers base their conclusions are varied and indefinite. All describe skin manifestations, but each differs markedly from the others. Thus, of the two sets of observers who used chimpanzees, Grünbaum speaks of a "doubtful

roseola not having a scarlatiniform appearance," while Landsteiner, Levaditi, and Prasek describe an "exanthem of tiny red spots and confluent areas." Cantacuzène and Bernhardt, working with the lower monkeys, present equally diverse descriptions of the rash. The former writes of "a uniform purple eruption on forehead, face and sometimes on the arms," which faded in thirty-six hours; whereas Bernhardt reports simply a "blotchy redness." Here is a striking dissimilarity in the description of a symptom which, from our present knowledge of the disease, must be looked upon as one of the most important. The chimpanzees are reported by both sets of observers to have shown a white exudate on the tonsils associated with an intense angina. Grünbaum does not mention the appearance of the tongue, though Landsteiner and his associates report that the tongues of their animals were red with large follicles. No throat involvement in the lower monkeys is mentioned by Cantacuzène or Bernhardt, and the latter alone mentions that his *Macacus rhesus* showed the typical coated raspberry tongue. On the question of desquamation Cantacuzène and Bernhardt agree that a large flaked scaling occurred, especially of the face and head, but Grünbaum and Landsteiner do not mention it. All the authors report rises of temperature to 40° and 41° C.

The symptoms described by these writers as evidences of scarlet fever in the monkey are not convincing (table I). They consist of variable moderate rises of temperature, irregular erythemas, and desquamation. Throat changes are described only by those who used chimpanzees. The question arises, therefore, whether any other test can be invoked to determine whether a specific infection has been accomplished. If we admit for the moment that the outspoken symptoms following some of the inoculations are evidences of a scarlatinal infection, it might be held that an immunity to reinoculation may be produced that furnishes, in doubtful instances, a means of determining whether a previous specific infection existed or not. In the only instance in which this test was applied, however, no immunity was demonstrated (Landsteiner, Levaditi, and Prasek).

But this is debatable ground. Analogies exist between the indefinite instances of so called specific infection in the monkey and

TABLE I.

	Temperature.	Rash.	Desquamation.	Throat.	Tongue.	Glands.
Grün- baum	39° C.	Doubtful roseola not having scar- latiniform ap- pearance.		White spots becoming an exudate on both tonsils. Streptococcus isolated from throat.		
Canta- cuzène	Rise to 40° C. Fluctuated for 23 days between 40° and 41° C.	Uniform purple eruption on forehead, face, and sometimes on forearms. Paled at end of 36 hours.	At end of 36 hours, large flaked des- quamation of face. Small scales on back, head, neck, and extremi- ties.			General adeno- pathy per- sisting for a long time.
Bernhardt	Normal, 38° C. Highest 40° C. Grad- ual fall.	Blotchy red- ness on face, shoulders, and neck.	Scaling; large flakes, includ- ing palms and soles.		Coated raspberry.	General swelling.
Land- steiner, Levaditi, and Prasek	38° to 40° C.	Exanthem of tiny red spots and confluent areas on neck, head, and ex- tremities.		Intense an- gina of ton- sils, pillars, and mucous membrane. White deposit on tonsils.	Red with large fol- licles.	

mild and almost unrecognizable cases of scarlet fever in man. However, in an experimental investigation of this nature only definite evidences of illness can be admitted as evidences of infection.

The quest is complicated by another factor. The causative agent of scarlet fever is still undiscovered. Its detection cannot, therefore, be utilized as evidence of infection. On the other hand, the streptococcus is a constant attendant of the scarlatinal infection; it exists not only in the nasopharynx, but often in the blood also. This microorganism may produce its own effects when materials containing it or its growth products are injected. To distinguish between the effects of the streptococcus and of the unknown agent of scarlet fever offers, therefore, another theoretical difficulty.

These considerations led us to employ merely the most prominent

symptoms in the monkey as evidences of possible infection, and no single sign, such as local erythema, slight rise in temperature, vomiting, or desquamation. Even when two or three signs coincided, they were taken to be suggestive rather than demonstrative. Viewed in this way, the import of the effects upon lower monkeys of the inoculation of materials from scarlet fever patients does not confirm the observations of Cantacuzène, Bernhardt, and others.

#### THE SOURCE AND NATURE OF THE INOCULATING MATERIALS EMPLOYED.

The materials employed for inoculation were derived from patients in the early stages of scarlet fever. This was rendered possible by the coöperation of the diagnosticians of the Department of Health. Several cases were received in the hospital within twelve hours, and many within twenty-four to thirty-six hours after the first appearance of the rash.

The materials used may be divided into three classes as follows: (1) artificially sterilized; (2) fresh unsterile; (3) fresh sterile.

The artificially sterilized material consisted of sputum treated with carbolic acid, so that the mixture contained 0.5 per cent. of the disinfectant. This combination was shaken in a machine for one half to one hour, then put in the thermostat for a few hours and shaken occasionally by hand. Sometimes in twenty-four, and usually in thirty-six hours, cultures from the mixture on glucose agar were sterile. This procedure has been shown by Flexner to destroy the bacteria contaminating poliomyelitic virus, without injuring the virus. After several experiments it became evident that the monkeys were highly resistant to infection by the microorganisms in the nasopharyngeal and ear secretions of human beings. Consequently the carbolized material was soon abandoned and fresh substances were transferred directly from patient to animal.

The fresh unsterile material consisted of (a) whole sputum shaken up with enough salt solution to render it evenly emulsified, (b) tongue and throat scrapings similarly shaken, (c) absorbent cotton tampons from the nasopharynx, (d) finely chopped tonsils, (e) washings from extirpated tonsils, (f) discharge from the ear.

This unsterile material was injected subcutaneously, submucously, and intraperitoneally without serious effect. Indeed, with the exception of sputum from a single individual, a general reaction was never observed, and in only two or three instances did local abscesses develop. Cultures from the pus of the abscesses almost invariably showed *Staphylococcus aureus*, and in no instance was the predominating organism of the injection mass recovered.

The third class of scarlatinous material was composed of (a) urine obtained under sterile precautions and found sterile by culture; (b) whole blood collected from a vein in paraffin-coated iced tubes, and injected, before clotting, intravenously into a monkey; (c) defibrinated blood; (d) sputum filtrates (Berkefeld); (e) filtrates of broth cultures of streptococcus from scarlatinous throats; (f) blood from scarlet fever cases mixed with ascitic broth and incubated at 37° C.

#### OBSERVATION OF THE INOCULATED MONKEYS.

The routine observation of the inoculated monkeys consisted in temperature readings twice daily, frequent blood counts, and physical examinations. Control observations were made on several normal monkeys over a period of about ten days before the regular inoculation series was begun. As a result of these preliminary findings, normal monkey temperature curves were considered unsatisfactory standards by which to measure pathological variations. Indeed we attached little significance to the irregularities of temperature which the animals showed, even when the readings were as high as 41° C. In several instances after a few days of rather high, irregular temperature the curve fell and continued almost level (Ringtail 2, *Cercocebus fuliginosus* 3). Occasionally, also, a temperature curve might show a sudden sharp rise for which no explanatory physical sign or treatment could be determined.

In contradistinction to the numerous irregularities in the temperature of stock animals were the remarkably even curves of many inoculated monkeys. For example, *Macacus rhesus* monkeys 10 and 12 received respectively five and four inoculations, yet their temperature curves were almost undisturbed. Still more striking

is the case of *Cynocephalus babuin* 16, who developed a purulent panophthalmitis, necessitating enucleation of the eyeball; yet, although his temperature was irregular, at no time did it rise above 40° C. Another paradoxical group was formed by the animals that began with a high temperature and whose curve sank gradually but steadily after inoculation (*Macacus rhesus* monkeys 29 and 30, and *Macacus nemestrinus* 20). In the case of *Macacus nemestrinus* 20 the lowest part of the temperature curve corresponded with the time when suppuration of the thighs existed. *Macacus rhesus* 25, on the other hand, displayed a distinct rise of temperature when abscesses formed in the groin.

These examples show how uncertain a criterion the temperature of the monkeys is. The blood counts were still more unsatisfactory and were given up early in the work.

A thorough physical examination of the animals was made every morning, and suspicious signs were watched during the day. It soon became evident that the superficial lymph nodes would be of little value, because in almost every animal they were enlarged, especially the axillary nodes. This was a constant finding. Furthermore, many of the animals, especially the *Macacus rhesus* monkeys, developed from time to time purplish red erythemas of varying intensity on the face and neck. In the case of one female with an unusually brilliant color about the buttocks, these erythematous blotches appeared in association with an intensification of the buttock redness. At the same time there was a bloody, mucous, vaginal discharge. The *Macacus nemestrinus* monkeys were much paler and in no case showed any redness. The *Macacus rhesus* monkeys likewise exhibited almost constantly a fine, bran-like desquamation, seen more especially over the neck, shoulders, and chest. It is difficult to see the tonsils in monkeys, for they are small and low down, and the faucial ring is narrow. In other respects the throat is easy to observe and in our series was always found to be rather pale with a slightly livid tinge. The tongue of monkeys is usually clean, though at times an irregularly light whitish coat may be seen. The papillæ tend to be prominent and pink.

## EXPERIMENTAL OBSERVATIONS.

The experiments were varied as much as possible, both with respect to the character of material and the site and method of inoculation, on the one hand, and the conditions bearing upon the susceptibility of the animals, on the other.

The routes of introduction were through the mucous membranes, skin, stomach, joint cavities, blood stream, peritoneum, and brain. It seemed possible that repeated inoculations at three week intervals over a long period might bring about a state of hypersensitiveness and so lead to infection. Consequently several monkeys were treated in that manner. The animals stood the inoculation well and showed no reaction of any kind. In this series most of the inoculations were made by swabbing the scarified mucoſa with infected material, and by submucous injections in the throat and nose with a hypodermic needle.

With the view of adapting a virus to an unusual host, two series of experiments were undertaken, in which infected material was injected subcutaneously in a region whose lymphatic apparatus was easily accessible for removal. The groin was used in each series, but the inoculation material differed. Series A received subcutaneously the supernatant fluid from an emulsion of fresh tonsils from scarlet fever patients, whereas series B was given an emulsion made from tongue scrapings. It is interesting to note that after the first passage the inguinal lymph nodes were sterile, although in some cases enlarged.

The first three animals of series A were treated as described above and the inguinal nodes were removed and an emulsion made from them was reinjected into another monkey. The last three animals of this series were treated in a similar way, except the final one, which received the emulsion of inguinal nodes partly in the brain and partly in the peritoneum (*Macacus rhesus* 28). Series B was a repetition of Bernhardt's method. Both series were negative.

Clinical experience suggests that scarlet fever and measles are not infrequently associated. This may depend purely upon coincidence, but the fact suggested to us the possibility that one disease might predispose to the other. Consequently, encouraged by the

successful reports by Anderson and Goldberger of the transmission of measles to *Macacus rhesus* monkeys we attempted to produce this disease in an animal which was to be inoculated later with scarlatinous material. Neither disease developed in the two animals injected.

The high degree of resistance shown by these animals led us to try two other modes of infection. The first was designed to overwhelm the animals with large, rapidly repeated inoculations of the infected material, and the second to employ methods which are known to lower the resistance of animals to infection. The following protocol illustrates the methods used.

A healthy *Macacus rhesus* monkey, giving a negative von Pirquet tuberculin test, was inoculated as follows:

February 23. 3 c.c. of defibrinated blood from a patient one day ill, intracerebrally; 25 c.c. of defibrinated blood from a patient one day ill, intraperitoneally.

February 24. 20 c.c. of an emulsion of inguinal nodes from *Macacus rhesus* 24 (previously inoculated), subcutaneously in the right groin and thigh.

February 25. 43 c.c., intraperitoneally, of a mixture made of 30 c.c. of fresh blood from a twenty-four hour old case of scarlet fever, incubated in 75 c.c. of ascitic broth at 37° C. for forty-five hours.

February 27. The throat, pharynx, and tongue were scarified and swabbed with fresh throat swabbings of a five day old case of scarlet fever.

March 2. 95 c.c., intraperitoneally, of a Berkefeld filtrate of the following materials: sodium chloride emulsion of fresh sputum of a five day old case of scarlet fever plus two broth flask cultures of streptococcus from the throat of a scarlet fever patient.

Notwithstanding this amount of inoculation, the animal showed no rise of temperature above 40° C. and no signs of illness or discomfort.

To diminish the resistance of the animals, the states of exsanguination and of anaphylactic shock were produced. In the first instance the animal was bled until severe symptoms of hemorrhage appeared and then a saline infusion was given. Following this, 50 c.c. of fresh whole blood were transferred directly from the vein of a twenty-four hour old case of scarlet fever into the circulation of the monkey. On the next day the monkey's throat and pharynx were swabbed with material from the throat of the same case that had supplied the blood. No symptoms developed. The last monkey treated was given a mild anaphylactic shock by injection of horse serum at proper intervals. On the day before the shock the animal received intracerebrally 2 c.c. of defibrinated blood from a forty-eight hour old case of scarlet fever. Four hours after the shock 3.5 c.c. of the same defibrinated blood were inoculated intracerebrally and 15 c.c. intraperitoneally. The pharynx was also swabbed with throat swabbings from the same case. No symptoms developed.



In the case of the exsanguinated monkey it is conceivable that the saline infusion acted as a diluent and thereby lowered still further the defensive mechanism of the body, already partially depleted of blood. Just how much the anaphylactic shock given to monkey 29 lowered its resistance to infection is problematical, especially in view of the fact that the blood used for inoculation of the brain and peritoneum contained streptococci. The animal developed no symptoms.

#### CONCLUSIONS.

1. The reported successful transfer of scarlet fever to both higher and lower monkeys is not definitely established.

2. In the course of the experiments here reported, the infectious agent can be assumed to have been carried over to the monkeys. The failure to cause infection probably proceeds from the insusceptibility of the monkeys employed, or to the manner of introducing the agent.

3. The temperature curve and leucocyte count of monkeys are unsatisfactory criteria for the diagnosis of disease in those animals.

4. Monkeys frequently have transient blotchy, erythematous eruptions on the face and neck, and almost always a bran-like desquamation.

5. Monkeys are highly resistant to infection with microorganisms from human beings.

#### BIBLIOGRAPHY.

- Anderson, J. F., and Goldberger, J., *Public Health Reports*, 1911, xxvi, 847, 887.  
Bernhardt, G., *Deutsch. med. Wchnschr.*, 1911, xxxvii, 791; *Centralbl. f. Bakteriol., 1te Abt., Ref.*, 1911, I, Suppl., 27.  
Cantacuzène, J., *Compt. rend. Soc. de biol.*, 1911, lxx, 403.  
Grünbaum, A. S., *Brit. Med. Jour.*, 1904, i, 817.  
Hektoen, L., and Weaver, G. H., *Jour. Am. Med. Assn.*, 1911, lvi, 1795.  
Landsteiner, K., Levaditi, C., and Prasek, E., *Ann. de l'Inst. Pasteur*, 1911, xxv, 754.  
Stickler, W., *New York Med. Rec.*, 1899, lvi, 363.

## THE POST-MORTEM EXAMINATION OF HORSES' HEARTS FROM CASES OF AURICULAR FIBRILLATION.

By ALFRED E. COHN.

*(From the Hospital of The Rockefeller Institute for Medical Research, New York.)*

This report describes the histological findings of the hearts of three horses known to have been the subjects of auricular fibrillation. They were reported in this Journal by Dr. Th. Lewis.<sup>3</sup> The hearts were fixed in Müller-Formal (9-1), washed in running water, preserved in 70 per cent. alcohol and shipped to New York. The tissues supposed to contain the sino-auricular node and the septum of the heart containing the conduction system were excised. The latter, being too large a block to embed *in toto* was cut in slices about 3 mm. thick in a plane at right angles to the long axis of the heart. The slices and the sinus bearing areas were embedded in celloidin paraffin and cut in sections 10-15 micra thick. Every fifth section was mounted and stained with iron hæmatoxylin and Van Gieson's solution.

### HEART FROM HORSE NO. I.

This heart had been cut in pieces before I received it. The pieces supposed to contain the sino-auricular node were prepared in the manner described. Examination of the series showed that the node had not been included in the pieces excised. Inspection of the remaining portions of the heart, which had been sent with the excised pieces, showed that the area at the cavo-auricular junction at which the sino-auricular node is found had been cut in such a fashion that it appeared doubtful whether a successful reconstruction would be possible. After examining the sinus node of the other two hearts it was decided that further search for the sinus node in this horse would not be profitable.

The auriculo-ventricular node is found without trouble. It lies in a region well below the origin of the aorta. The central fibrous body of the human and canine heart is represented in horses, as in calves, by a cartilaginous structure. The node lies to the right of the central cartilage and is curved about its anterior extremity. In this horse it measures 3 mm. by 4 mm. in diameter. It has a distinct, well-developed auriculo-nodal junction, composed of fibres showing a transition from those forming the muscle of the auricle to those forming the muscle of the node. The auricular muscle requires no special description; it is similar to mammalian cardiac muscle. The structure of the *A-V* node offers certain peculiarities. It is formed of a meshwork of very coarse thick strands of connective tissue, supplied with a moderate number of nuclei. These meshes when magnified to about thirty diameters have proportions similar to fish netting. In the interstices of the network, the muscle fibres of the node are found. These are like Purkinje cells, in the number and arrangement of their muscle fibrillæ, and in the character and number of their nuclei. The cells are probably larger than those seen in sheep and calves' hearts.

The *A-V* node is continued to the anterior aspect of the central cartilaginous body, and passes into the main stem which is exceedingly short, and which soon divides into a right and a left branch. The septum ventriculorum is almost entirely muscular at the level at which the beginning of the conduction system lies; an analogue of the septum membranaceum, as it exists in the human heart, was not found. It is within a thick muscular wall therefore that the division into right and left branches takes place. These are, relatively speaking, very small, showing only three or four cells side by side in cross section. As far as these were followed they lie within the muscular wall of the septum.

At the levels at which the main stem divides, Purkinje fibres are seen lying under the endocardium of both ventricles. These are undoubtedly the recurrent branches, which pass upwards to the base of the heart from the level of the papillary muscles, being continuous at that point with the arborisations of the right and left branches of the main stem.

No lesion, inflammatory or otherwise, was discovered in the course of the conduction system.

#### HEART OF HORSE NO. 3.

The area corresponding to the *sulcus (tænia) terminalis* was excised and was divided horizontally into two pieces, on account of its length. About five hundred sections (6,000 micra) were examined and the node was found in all of them. No abnormality was found in this node. To avoid repetition, a full description of this structure is postponed. That given for the heart of horse No. 5, in which the node is similar, may serve for both.

A description of the conduction system would duplicate that given for the heart of horse No. 1, and may therefore also be omitted.

#### HEART OF HORSE NO. 5.

*The Sino-Auricular Node.*—The entire cavo-auricular junction of this heart was embedded. It included the circumference of the superior vena cava, the atrium below it, and the adjoining portions of both those walls of the right auricular appendix which unite to form its upper border. A block, 10 cm. deep, was cut into sections 10 micra thick, the plane of section being at right angles to the long axis of the vena cava. Every fifth section was mounted. It was found that this large piece had been so cut that it failed to contain the sinus node entirely. The lower levels of it were subsequently recovered in the portion of the anterior wall of the right auricular appendix lying to the right. No attempt has been made to calculate the size of the nodal tissue in these pieces. In the large piece the node was estimated to be 16.95 mm. (16,950 micra) long.

The upper extremity of the node is found in the wall of the right auricle above the entrance of the superior vena cava. It is embedded deep in the wall of the auricle midway between endocardium and pericardium. It gradually becomes differentiated from auricular muscle by the increase of connective tissue strands which appear among the muscle fibres. At a slightly lower level (1 mm.) the node assumes a more superficial position, lying 1 mm. from the pericardium. Its cross section is long and narrow, measuring 13

by 2 mm. The pericardium over it is much thicker than elsewhere. There are many large nerves and numerous ganglia in its neighbourhood. There is no special large vessel, but there are numerous small ones.

At a slightly lower level, 1.5 mm. from the upper extremity, the node divides into two (tails), which may be designated as anterior (representing the structure which is usually seen) and posterior. This division occurs just a little above the entrance of the superior vena cava into auricle. Each of these tails of the node measures 8 by 1 mm. in cross section; they separate rapidly so that an interval between them, at first of 3 mm., rapidly widens to 16 mm., 0.7 mm. below the division. The space between these two portions of the node is formed of closely meshed and thin connective tissue which contains a moderate amount of fat. Here the anterior tail measures 10 by 0.5 mm., the posterior one, 5 by 2 mm.. Both are approximately triangular in outline. The posterior tail is 7.1 mm. in length from the point of division to its end. The anterior tail is 16.95 mm. in length, and at the lowest point at which its cross section is observed, measures 8 by 0.5 mm.

The node contains more connective tissue than the surrounding auricular muscle with which it communicates freely, but the amount is not abnormal. The muscle of which it is composed shows the usual interlacement, and the numerous nuclei, which are seen in this structure in other mammals; but the muscle fibres themselves are slightly thicker and are less striated. The fibres have no likeness to Purkinje cells. There is no evidence of abnormality with the exception of a small group of round cells near the lower extremity of the posterior tail of the node.

The occurrence of a bifid sinus node has been described by Schwartz<sup>5</sup> in the calf and sheep. When the sections of the heart of horse No. 3 were cut, two years ago, the pieces were not so large as those taken since that time. The nature of the divided sinus node therefore escaped observation. In the heart of horse No. 5 the structure of the node was similar to that found by Schwartz.<sup>5</sup> The node may be described as having the form of an inverted Y, the stem of which is short, and the anterior limb of the bifurcation being longer than the posterior.

The *auriculo-ventricular node* corresponds in general structure and in topography to that found in horse No. 1. A few details only need be added. In excising the piece of the septum of this heart containing the conducting apparatus, the coronary sinus was left intact. The relation of the auriculo-ventricular node to it can therefore be ascertained. It lies in the anterior lip of the coronary sinus and its upper border is a few millimetres posterior to the line of attachment of the mesial cusp of the tricuspid valve. At this level the node measures 9 by 1 to 1.5 mm., a little lower it measures 11 by 2.25 mm., and lower still 9 by 1 mm..

The connective tissue meshes of the node in this, as in the other hearts, are only partly filled by muscle fibres. It is difficult to decide whether the spaces left are normal or are artifacts produced in the preparation of the sections. Opposed to the conclusion that they are artifacts is the unlikelyhood of Purkinje cells shrinking much when the surrounding ventricular muscle in the same section suffers little or at all. The Purkinje cells probably contain relatively more undifferentiated protoplasm than the muscle fibres, and this difference between them may render the deduction that one is unchanged because the other obviously is, unfair. If the spaces are not artifacts, they must be preformed. For this view there is some evidence. The spaces are lined with a membrane which presents slight nodular thickenings. These thickenings may represent cells, but they fail to show definite nuclear structure, at least with the dyes used in this investigation. Curran<sup>2</sup> would no doubt have identified these spaces with the bursæ he described. Another reason for thinking that such spaces are preformed is that they can all be filled by injecting fluid at a single appropriate place. This procedure has been successfully undertaken by Lhamon<sup>4</sup> and by the author.<sup>1</sup> It is probable that the spaces are preformed but their unusual size suggests shrinkage of the muscle cells within them.

The position, course, size and structure of the right and left branches are like those found in horse No. 1. No evidence of abnormality or of inflammation, old or recent, was observed.

## SUMMARY AND CONCLUSIONS.

The hearts of three horses, known to have suffered from complete irregularity of the heart, have been examined. In the first heart, the sino-auricular node was not found; in the other two, it showed no abnormality. The conduction system in all three hearts was intact and showed no lesion.

An arrangement of the sino-auricular node like that found in certain other mammals was observed in one of the hearts.

The auriculo-ventricular node, main stem and branches in these horses' hearts, are described in detail. They were free from abnormality.

## BIBLIOGRAPHY.

- <sup>1</sup> COHN (A. E.). "Demonstration of Ox-Hearts showing injection of Conduction System." *Proc. of the New York Pathol. Soc.*, 1911, *XI*, 130-132.
- <sup>2</sup> CURRAN (E. J.). "A constant bursa in relation with the bundle of His; with studies of the auricular connections of the bundle." *Anat. Anzeiger.*, 1910, *XXXV*, 89-97; *Anat. Record*, 1909, *III*, 618-632.
- <sup>3</sup> LEWIS (TH.). "Irregularity of the heart's action in horses and its relationship to fibrillation of the auricles in experiment and to complete irregularity of the human heart." *Heart*, 1912, *III*, 161-171.
- <sup>4</sup> LHAMON (R. M.). "The sheath of the sino-ventricular bundle." *Amer. Journ. of Anat.*, 1912, *XIII*, 55-70.
- <sup>5</sup> SCHWARTZ (G.). "Untersuchungen über das Sinusgebiet im Wiederkäuerherzen." *Inaug.-Dissert.*, Berlin, 1910. *Archiv. f. wissensch. u. prakt. Tierheilkunde*, 1910, *XXXVII*, 152-179.

## OBSERVATIONS ON INJECTION SPECIMENS OF THE CONDUCTION SYSTEM IN OX HEARTS.

By ALFRED E. COHN.

*(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)*

In his preface to Tawara's monograph on the conduction system of the heart, Aschoff pointed out that in performing its orderly function, the region of the papillary muscles should contract before those of the base of the heart. He was able to prove from Tawara's investigations that the atrio-ventricular bundle was in reality distributed to this region first and only later to the base itself. These conclusions harmonised certain views of the embryology and the physiology of the heart with those put forward by Tawara concerning its structure. The method of investigating the anatomical pathway for conduction attained success only when serial sections of the heart were made and the system reconstructed. This method is tedious and the interpretation difficult. Numerous investigations attempted the method of gross dissection to lay bare the atrio-ventricular bundle, but satisfactory dissections of so complicated a system are quite impossible on account of the nature of the bundle and its ultimate distribution. For it is probable that no portion of the heart is free from a lining of conducting fibres, at least of Purkinje fibres, and these cannot be followed by the method of dissection. And since the origin of these fibers cannot be ascertained in this way, a theory of the distribution of the impulse to contraction cannot properly be constructed from the knowledge so gained.

A method by which the conducting system can be brought into view with ease and without subjecting it to trauma is by injection.\*

\* For the suggestion that an injection of the heart is possible the author is indebted to Dr. W. G. MacCallum. Dr. MacCallum had seen specimens so prepared in the summer of 1911 at Stanford University.



Dyes were at first used as the injecting fluid. Some of the specimens demonstrated to the New York Pathological Society<sup>1</sup> were prepared in this manner. The stains that were used were all readily diffusible and were discarded on that account. Black india ink was then employed but was found to be too viscid to be a satisfactory medium, but india ink diluted with an equal part of water served the purpose very well. About twelve hearts in all were examined.

The ox hearts, of which photographs are shown (Fig. 1 and 2) were prepared in this way. After injection, these hearts were preserved in Kaiserling solution and have been kept as museum preparations. Lhamon<sup>2</sup> has published a photograph of the cavity of the left ventricle prepared in a similar way but none of the right. The photograph now published of the left ventricle is in close agreement with his. A photograph of a successful injection of the right ventricle is now also reproduced.

It is not the intention of the present communication to discuss purely structural or histological matters. Lhamon<sup>2</sup> has made observations on these subjects which the author has been able to substantiate. It is the purpose of this communication to point out the clearness with which these injected specimens display the course and distribution of the impulse of conduction in the two ventricles.

In the left ventricle, three main branches have been named: one to the anterior, one to the posterior papillary muscle and one to the apex, the last issuing by two heads from the preceding branches. A comparison of these with the branches of the right ventricle will disclose a striking similarity in the general arrangement on the two sides. In the right ventricle one branch passes to the large anterior papillary muscle and to the venous base of the right ventricle, and one along the border of the large moderator band to the large septal papillary muscle, and to the conus arteriosus; the branch to the apex issues by separate heads from both the preceding. Thus the arrangement in the right ventricle is very like that of the left, but differs from it, in that the branches which are easy to identify as single strands on the left side, appear on the right broken into groups of branches, having a more extensive interlacement.

The gradual filling out of all these spaces in an orderly fashion

is easily discernible during the progress of a successful injection. If the point of the fine injecting needle has been fortunately inserted in the left division of the bundle of the ventricle, the fluid can be seen to course parallelly along the three branches of the left division and to arrive at the papillary muscles first and a little later at the apex. The time relations are of course unreliable. But it is very important to notice that the injection of the papillary muscle takes place before that of the base, both venous and arterial. It is believed that inspection of the photograph demonstrates clearly that the injected pathways at the bases do not communicate with the left branch before division. They are in communication only with the branches supplied to papillary muscles; that is to say, the papillary muscles are supplied before either base or apex. This is the contention for which Aschoff argued. This method of distribution to the bases is true of the right ventricle as well as of the left.

Inspection during injection then shows that the papillary muscles are reached first by the fluid, and the bases and apex later. Whether the apex or one or the other base precede one another cannot be decided by this method; nor do the photographs aid in the decision; they show only the dependence of the basal portions on the papillary muscles for this distribution. Measurement of the relative length to each part from the origin of the left division may be useful in arriving at a solution, but such measurements have not been undertaken.

#### RESULTS AND CONCLUSION.

Injection by diluted india ink is a valuable and simple method for demonstrating the course and relations of the conduction system in ox hearts. It has been shown that the injection reaches the papillary muscles first, and other portions later, notably the bases and apex; and also that the branches of the system to these latter portions have no direct relationship with the main divisions or their principal branches. The basal and apical branches communicate with the principal branches at the papillary muscles.

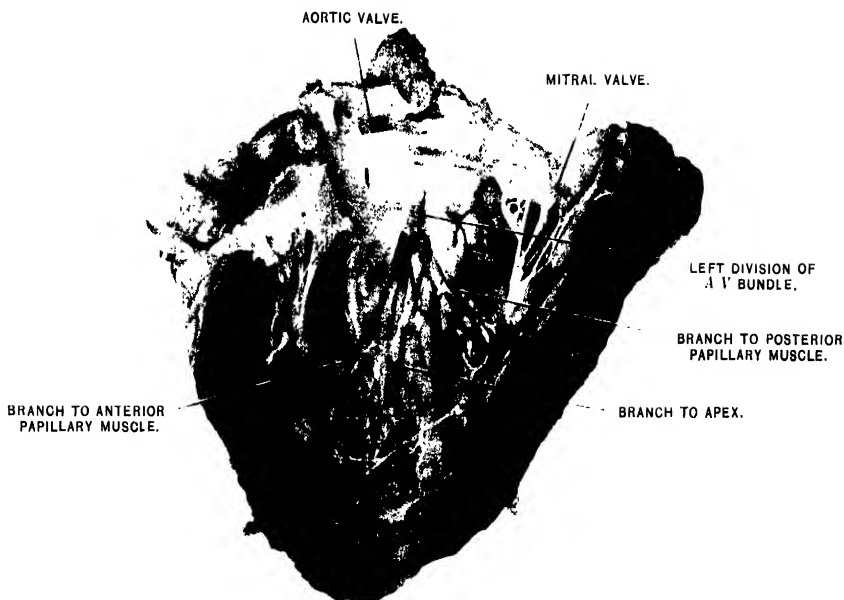
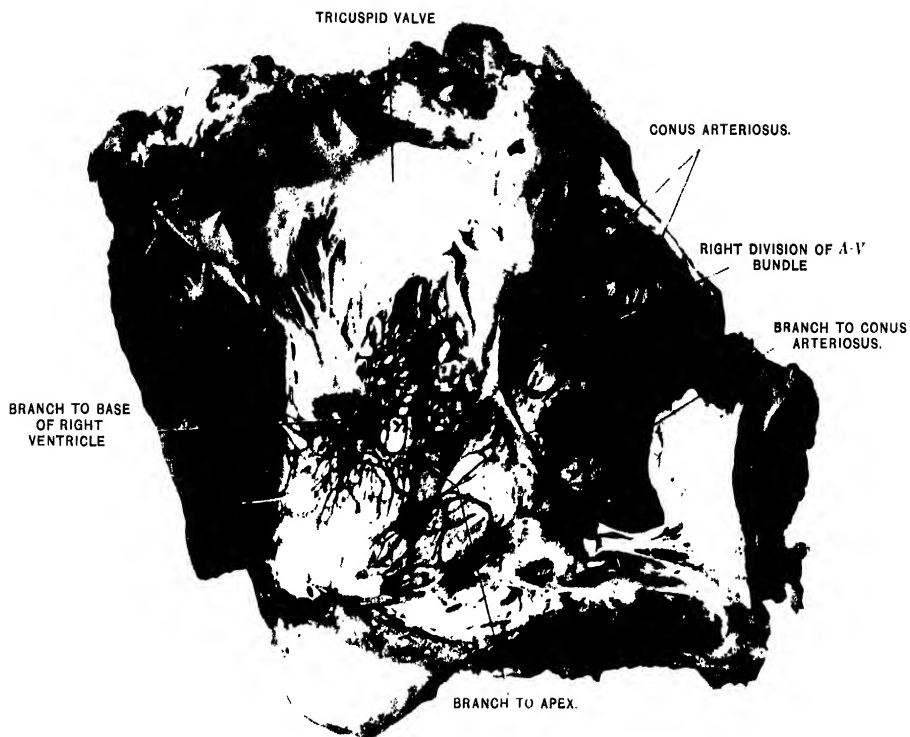
The contention of Aschoff in respect of the course of conduction through the heart is therefore substantiated by this method of investigation.

## BIBLIOGRAPHY.

- <sup>1</sup> COEN (A. E.). "Demonstration of Ox-Hearts showing injection of the Conduction System." Proc. of the New York Pathol. Soc., 1911, xi, 130-132.
- <sup>2</sup> LHAMON (R. H.). "The sheath of the sino-ventricular bundle" Amer. Journ. of Anat., 1912, xiii, 55-70.

Fig. 1. A photograph of the cavity of the left ventricle. Injection of the conduction system is shown.

Fig. 2. A photograph of the cavity of the right ventricle. Injection of the branches as in Fig. 1.





# INDEX.

- A** CID, cerebronic. Second paper, 329  
 Acid, lactic, formation of, from carbohydrates, mechanism of, 323  
 Arsenic, absorption of, following intramuscular injections of salvarsan and neosalvarsan, 541  
 Artificial parthenogenesis, comparative efficiency of weak and strong bases in, 373  
 Artificial respiration, effective, in emergencies, simple devices for, 306  
 AUER, JOHN, and MELTZER, S. J. Der afferente Splanchnikus als Depressor, 297  
 Auricles, faradized, in the dog's heart, influence of vagus nerves on, 554  
 Auricular fibrillation, post-mortem examination of horses' hearts from cases of, 579  
 AUSTIN, J. H. See PEARCE and AUSTIN, 81  
 —. See PEARCE, AUSTIN, and MUSSER, 59  
 Axis cylinders *in vitro*, degeneration and regeneration of, 350  
  
**B**ACTERIOLYSIN, leucocytic, relation of, to body fluids, 192  
 BANCROFT, F. W. See LOEB and BANCROFT, 461  
 Bases, strong and weak, comparative efficiency of, in artificial parthenogenesis, 373  
 Bases, weak and strong, relative influence of, upon the rate of oxidations in the unfertilized egg of the sea urchin, 487  
 BEUTNER, REINHARD. Die physikalische Natur bioelektrischer Potentialdifferenzen, 386  
 —. Einige weitere Versuche betreffend osmotische und kolloidale Quellung des Muskels, 453  
 —. New galvanic phenomena, 463  
 —. New electric properties of a semipermeable membrane of copper ferrocyanide, 472  
 Bioelektrischen Potentialdifferenzen, physikalische Natur der, 386  
 Blood, action of pneumococcus on, 277  
 Blood, changes in, following splenectomy, and their relation to hemolytic jaundice, 59  
 Blood corpuscles, red, color index and color of, 283  
 Blood corpuscles, red, increased resistance of, after splenectomy, 70  
 Blood destruction and regeneration and hemolytic jaundice, relation of the spleen to, 59, 70, 81  
 Blood, nitrogen retention in, in experimental acute nephritis of the cat, 90  
 Bone and cartilage produced by a transplantable new growth of the fowl, 207  
 Brain in cases of general paralysis, a demonstration of *Treponema pallidum* in, 151  
 Brain, sulphatide of, 318  
 BRONFENBRENNER, J., and NOGUCHI, H. On the resistance of various spirochaetes in cultures to the action of chemical and physical agents, 185  
 BUTTERFIELD, E. E. The reaction between oxygen and hemoglobin, 158  
 —. The color index and color of the red blood corpuscles, 283  
 BUTTERFIELD, E. E., and PEABODY, FRANCIS W. The action of pneumococcus on blood, 277  
  
**C**ALCIUM and magnesium, inorganic metabolism in pneumonia with especial reference to, 529  
 Calcium excretion, excessive, through the feces in infantilism, cause of, 524  
 Calligyrum, *Treponema* (new species), from condylomata of man, cultivation of, 121  
 Cancerous and normal epithelium, false transitions between, 235  
 Carbohydrates, mechanism of lactic acid formation from, 323  
 CARREL, ALEXIS. Visceral organisms, 341  
 —. Artificial activation of the growth *in vitro* of connective tissue, 344

- Cartilage and bone produced by a transplantable new growth of the fowl, 207
- Cells, endothelial, of the lymph nodes and liver, changes in, in splenectomized animals receiving hemolytic serum, 81
- Cerebronic acid. Second paper, 329
- CHAMBERS, R. See NOVY, PERKINS, and CHAMBERS, 35
- Chemical and physical agents, resistance of various spirochætes in cultures to the action of, 185
- CLARK, PAUL F. See FLEXNER and CLARK, 272
- See FLEXNER, CLARK, and FRASER, 131
- See HOWARD and CLARK, 98
- COHN, ALFRED E. On the differences in the effects of stimulation of the two vagus nerves on rate and conduction of the dog's heart, 494
- The post-mortem examination of horses' hearts from cases of auricular fibrillation, 579
- Observations on injection specimens of the conduction system in ox hearts, 585
- Color and color index of the red blood corpuscles, 283
- Color index and color of the red blood corpuscles, 283
- Complement-fixation reaction, application of, to the diphtheria group of organisms, 51
- Conduction system in ox hearts, observations on injection specimens of, 585
- Condylomata of man, cultivation of *Treponema calligyrum* (new species) from, 121
- Connective tissue, artificial activation of growth of, *in vitro*, 344
- Connective tissue, permanent life of, outside of the organism, 360
- Copper ferrocyanide, new electric properties of a semipermeable membrane of, 472
- Corpuscle, red, extracellular relation of malarial parasite to, and its method of securing attachment to the external surface of the red corpuscle, 165
- Corpuscles, red blood, color index and color of, 283
- Corpuscles, red blood, increased resistance of, after splenectomy, 70
- Cultivation of the virus of poliomyelitis, experiments on, 136
- Cultivation of *Treponema calligyrum* (new species), from condylomata of man, 121
- Cultures of *Trypanosoma lewisi*, immunization by means of, 35
- Cultures of various spirochætes, resistance of, to the action of chemical and physical agents, 185
- DENIS, W. See FOLIN, KARSNER, and DENIS, 90
- Diphtheria group of organisms, application of complement-fixation reaction to, 51
- DRAPER, GEORGE, and HANFORD, JOHN M. Experiments on the transmission of scarlet fever to the lower monkeys, 569
- EBELING, ALBERT H. The permanent life of connective tissue outside of the organism, 360
- Egg, unfertilized, of the sea urchin, relative influence of weak and strong bases upon the rate of oxidations in, 487
- Electric properties, new, of a semipermeable membrane of copper ferrocyanide, 472
- Elektrolytwirkung, antagonistische, und Permeabilität, nach einer neuen Methode, 410
- Embryo of foreign species, transplantability of tissues to, 223
- Endothelial cells of the lymph nodes and liver, changes in, in splenectomized animals receiving hemolytic serum, 81
- Epidemic meningitis, results of serum treatment in thirteen hundred cases of, 248
- Epidemic poliomyelitis. Fourteenth note: Passive human carriage of the virus of poliomyelitis, 131
- Epithelium, cancerous and normal, false transitions between, 235
- Experimental acute nephritis of the cat, nitrogen retention in the blood in, 90
- Experimental tuberculous meningitis, effects of subdural injections of leucocytes on development and course of, 108
- Experiments on the cultivation of the virus of poliomyelitis, 136
- Experiments on the transmission of scarlet fever to the lower monkeys, 569
- Extracellular relation of the malarial parasite to the red corpuscle, and its method of securing attachment to

the external surface of the red corpuscle, 165

**FALES, H. L.** See MCCRUDDEN and FALES, 520, 524, 547, 550

Faradized auricles in the dog's heart, influence of vagus nerves on, 554

Feces in infantilism, cause of excessive calcium excretion through, 524

Feces in infantilism, nature and origin of nitrogenous compounds in, 520

Ferrocyanide, copper, new electric properties of a semipermeable membrane of, 472

Fibrillation, auricular, post-mortem examination of horses' hearts from cases of, 579

Filterable agent the cause of a second chicken tumor, an osteochondrosarcoma, 30

Filterable agent, variations in a chicken sarcoma caused by, 138

Fixation of complement reaction, application of, to the diphtheria group of organisms, 51

**FLEXNER, SIMON.** Some problems in infection and its control, I

— The results of the serum treatment in thirteen hundred cases of epidemic meningitis, 248

**FLEXNER, SIMON, and CLARK, PAUL F.** Paralysis in a dog simulating poliomyelitis, 272

**FLEXNER, SIMON, CLARK, PAUL F., and FRASER, FRANCIS R.** Epidemic poliomyelitis. Fourteenth note: Passive human carriage of the virus of poliomyelitis, 131

**FLEXNER, SIMON, and NOGUCHI, HIDEYO.** Experiments on the cultivation of the virus of poliomyelitis. Fifteenth note, 136

Fluids of body, relation of leucocytic bacteriolysin to, 192

**FOLIN, OTTO, KARSNER, HOWARD T., and DENIS, W.** Nitrogen retention in the blood in experimental acute nephritis of the cat, 90

**FRASER, FRANCIS R.** See **FLEXNER, CLARK, and FRASER, 131**

**GALVANIC** phenomena, new, 463

**HANFORD, JOHN M.** See **DRAPER and HANFORD, 569**

Heart of the dog, differences in effects of stimulation of the two vagus nerves on rate and conduction of, 494

Heart of the dog, influence of vagus nerves on faradized auricles in, 554

Hearts of horses from cases of auricular fibrillation, post-mortem examination of, 579

Hearts of oxen, observations on injection specimens of the conduction system in, 585

Hemoglobin and oxygen, reaction between, 158

Hemolytic jaundice and blood destruction and regeneration, relation of the spleen to, 59, 70, 81

Hemolytic jaundice, relation to, of changes in blood following splenectomy, 59

Hemolytic serum, changes in the endothelial cells of the lymph nodes and liver in splenectomized animals receiving, 81

Hexoses and pentoses, action of leucocytes on some, 323

**HOWARD, C. W., and CLARK, PAUL F.** Experiments on insect transmission of the virus of poliomyelitis, 98

**IMMUNITY, Tumor-, and tissue specificity, 223**

Immunization by means of cultures of *Trypanosoma lewisi*, 35

Immunology, a study of increased resistance of red blood corpuscles after splenectomy, by methods of, 70

*In vitro* artificial activation of growth of connective tissue, 344

*In vitro* degeneration and regeneration of axis cylinders, 350

Index, Color-, and color of the red blood corpuscles, 283

Infantilism, cause of excessive calcium excretion through feces in, 524

Infantilism, cause of failure to develop in, 550

Infantilism, intestinal absorption in, 547

Infantilism, nature and origin of nitrogenous compounds in feces in, 520

Infection and its control, some problems in, I

**INGEBRIGTSEN, RAGNVALD.** Studies of the degeneration and regeneration of axis cylinders *in vitro*, 350

Injection specimens of the conduction system in ox hearts, observations on, 585

Injections, intramuscular, of salvarsan and neosalvarsan, arsenic absorption following, 541

Injections, subdural, of leucocytes, effects of, on development and



- course of experimental tuberculous meningitis, 108
- Inorganic metabolism in pneumonia with especial reference to calcium and magnesium, 529
- Insect transmission of the virus of poliomyelitis, 98
- Insufflation, intrabronchial, of killed virulent pneumococci and of plain sterile bouillon, reaction of the lungs to, 301
- Insufflation, intrabronchial, of non-virulent pneumococci, pneumonic lesions made by, 288
- Intestinal absorption in infantilism, 547
- Intrabronchial insufflation of killed virulent pneumococci and plain sterile bouillon, reaction of the lungs to, 301
- Intrabronchial insufflation of non-virulent pneumococci, pneumonic lesions made by, 288
- Intramuscular injections of salvarsan and neosalvarsan, arsenic absorption following, 541
- J**AUNDICE, hemolytic, and blood destruction and regeneration, relation of the spleen to, 59, 70, 81
- Jaundice, hemolytic, relation to, of changes in blood following splenectomy, 59
- K**ARSNER, HOWARD T., and PEARCE, RICHARD M. The relation of the spleen to blood destruction and regeneration and to hemolytic jaundice. IV. A study, by the methods of immunology, of the increased resistance of the red blood corpuscles after splenectomy, 70
- KARSNER, HOWARD T. See FOLIN, KARSNER, and DENIS, 90
- Kolloidale und osmotische Quellung des Muskels, 453
- L**ACTIC acid formation from carbohydrates, mechanism of, 323
- LA FORGE, F. B. See LEVENE and LA FORGE, 320
- LAWSON, MARY ROWLEY. The extracellular relation of the malarial parasite to the red corpuscle, and its method of securing attachment to the external surface of the red corpuscle, 165
- Lesions, pneumonic, made by intrabronchial insufflation of non-virulent pneumococci, 288
- Leucocytes, action of, on some hexoses and pentoses, 323
- Leucocytes, effects of subdural injections of, on development and course of experimental tuberculous meningitis, 108
- Leucocytic bacteriolysin, relation of, to body fluids, 192
- LEVENE, P. A. The sulphatide of the brain, 318
- On the components of sphingomyelin, 339
- LEVENE, P. A., and LA FORGE, F. B. On nucleases. Third paper, 320
- LEVENE, P. A., and MEYER, G. M. On the action of leucocytes on some hexoses and pentoses. Third communication. Contribution to the mechanism of lactic acid formation from carbohydrates, 323
- LEVENE, P. A., and WEST, C. J. On cerebronic acid. Second paper, 329
- Lewis, *Trypanosoma*, immunization by means of, 35
- Liver and lymph nodes, changes in the endothelial cells of, in splenectomized animals receiving hemolytic serum, 81
- LOEB, JACQUES. The comparative efficiency of weak and strong bases in artificial parthenogenesis, 373
- Untersuchungen über Permeabilität und antagonistische Elektrolytwirkung nach einer neuen Methode, 410
- LOEB, JACQUES, and BANCROFT, F. W. The sex of a parthenogenetic tadpole and frog, 461
- LOEB, JACQUES, and WASTENEYS, HAROLD. Die Oxydationsvorgänge im befruchteten und unbefruchteten Seesternei, 450
- The relative influence of weak and strong bases upon the rate of oxidations in the unfertilized egg of the sea urchin, 487
- Lungs, reaction of, to the intrabronchial insufflation of killed virulent pneumococci and of plain sterile bouillon, 301
- Lymph nodes and liver, changes in the endothelial cells of, in splenectomized animals receiving hemolytic serum, 81
- M**AGNESIUM and calcium, inorganic metabolism in pneumonia with especial reference to, 529
- Malarial parasite, extracellular relation of, to the red corpuscle, and its method of securing attachment to

- the external surface of the red corpuscle, 165
- MANWARING, WILFRED H. The effects of subdural injections of leucocytes on the development and course of experimental tuberculous meningitis. Second paper, 108
- . The relation of leucocytic bacteriolysin to body fluids, 192
- MCCRUDEN, F. H., and FALES, H. L. The nature and origin of the nitrogenous compounds in the feces in infantilism, 520
- . The cause of the excessive calcium excretion through the feces in infantilism, 524
- . Intestinal absorption in infantilism, 547
- . The cause of the failure to develop in infantilism, 550
- MELTZER, S. J. Simple devices for effective artificial respiration in emergencies, 306
- . See AUER and MELTZER, 207
- . See WOLLSTEIN and MELTZER, 288, 301
- Membrane, semipermeable, of copper ferrocyanide, new electric properties of, 472
- Meningitis, epidemic, results of serum treatment in thirteen hundred cases of, 248
- Meningitis, experimental tuberculous, effects of subdural injections of leucocytes on development and course of, 108
- Metabolism, inorganic, in pneumonia, with especial reference to calcium and magnesium, 529
- MEYER, G. M. See LEVENE and MEYER, 323
- MOORE, J. W. See NOGUCHI and MOORE, 151
- MORSE, MARY ELIZABETH. The application of the complement-fixation reaction to the diphtheria group of organisms, 51
- MURPHY, JAMES B. Transplantability of tissues to the embryo of foreign species. Its bearing on questions of tissue specificity and tumor immunity, 223
- . See ROUS and MURPHY, 138
- . See ROUS, MURPHY, and TYTLER, 30
- Muskels, osmotische und kolloidale Quellung des, 453
- MUSSER, JOHN H., JR. See PEARCE, AUSTIN, and MUSSER, 59
- NEOSALVARSAN and salvarsan, arsenic absorption following intramuscular injections of, 541
- Nephritis, experimental acute, of the cat, nitrogen retention in the blood in, 90
- Nerves, vagus, differences in effects of stimulation of, on rate and conduction of the dog's heart, 494
- Nerves, vagus, influence of, on faradized auricles in the dog's heart, 554
- Nitrogen retention in the blood in experimental acute nephritis of the cat, 90
- Nitrogenous compounds in feces in infantilism, nature and origin of, 520
- Nodes, lymphatic, and liver, changes in the endothelial cells of, in splenectomized animals receiving hemolytic serum, 81
- NOGUCHI, HIDEYO. Cultivation of *Treponema calligyrum* (new species) from condylomata of man, 121
- . Studien über den Nachweis der *Spirochata pallida* im Zentralnervensystem bei der progressiven Paralyse und bei Tabes dorsalis, 239
- NOGUCHI, HIDEYO, and MOORE, J. W. A demonstration of *Treponema pallidum* in the brain in cases of general paralysis, 151
- NOGUCHI, HIDEYO. See BRONFENBRENNER and NOGUCHI, 185
- . See FLEXNER and NOGUCHI, 136
- NOVY, F. G., PERKINS, W. A., and CHAMBERS, R. Immunization by means of cultures of *Trypanosoma lewisi*, 35
- Nucleases. Third paper, 320
- ORGANISM, permanent life of connective tissue outside of, 360
- Organisms, diphtheria group of, application of complement-fixation reaction to, 51
- Organisms, visceral, 341
- Osmotische und kolloidale Quellung des Muskels, 453
- Osteochondrosarcoma, a second chicken tumor caused by a filterable agent, 30
- Oxidations, relative influence of weak and strong bases upon the rate of, in the unfertilized egg of the sea urchin, 487
- Oxydationsvorgänge im befruchteten und unbefruchteten Seesternei, 450
- Oxygen and hemoglobin, reaction between, 158

- PALLIDA**, *Spirochæta*, Studien über den Nachweis der, im Zentralnervensystem bei der progressiven Paralyse und bei Tabes dorsalis, 239
- Pallidum*, *Treponema*, demonstration of, in brain in cases of general paralysis, 151
- Paralyse, progressiver, und Tabes dorsalis, Studien über den Nachweis der *Spirochæta pallida* im Zentralnervensystem bei, 239
- Paralysis, general, demonstration of *Treponema pallidum* in the brain in cases of, 151
- Paralysis in a dog simulating poliomyelitis, 272
- Parasite, malarial, extracellular relation of, to red corpuscle, and its method of securing attachment to the external surface of the red corpuscle, 165
- Parthenogenesis, artificial, comparative efficiency of weak and strong bases in, 373
- Parthenogenetic tadpole and frog, sex of, 461
- PEABODY, FRANCIS W. Studies of the inorganic metabolism in pneumonia with especial reference to calcium and magnesium, 529
- See BUTTERFIELD and PEABODY, 277
- PEARCE, RICHARD M., AUSTIN, J. H., and MUSSER, JOHN H., JR. The relation of the spleen to blood destruction and regeneration and to hemolytic jaundice. III. The changes in the blood following splenectomy and their relation to the production of hemolytic jaundice, 59
- PEARCE, RICHARD M., and AUSTIN, J. H. The relation of the spleen to blood destruction and regeneration and to hemolytic jaundice. V. Changes in the endothelial cells of the lymph nodes and liver in splenectomized animals receiving hemolytic serum, 81
- PEARCE, RICHARD M. See KARSNER and PEARCE, 70
- Pentoses and hexoses, action of leucocytes on some, 323
- PERKINS, W. A. See NOVY, PERKINS, and CHAMBERS, 35
- Permeabilität und antagonistische Elektrolytwirkung nach einer neuen Methode, 410
- Physical and chemical agents, resistance of various spirochætes in cultures to the action of, 185
- Physikalische Natur bioelektrischer Potentialdifferenzen, 386
- Pneumococci, killed virulent, and plain sterile bouillon, reaction of lungs to intrabronchial insufflation of, 301
- Pneumococci, non-virulent, pneumonic lesions made by intrabronchial insufflation of, 288
- Pneumococcus, action of, on blood, 277
- Pneumonia, inorganic metabolism in, with especial reference to calcium and magnesium, 529
- Pneumonic lesions made by intrabronchial insufflation of non-virulent pneumococci, 288
- Poliomyelitis, epidemic. Fourteenth note: Passive human carriage of the virus of poliomyelitis, 131
- Poliomyelitis, experiments on the cultivation of the virus of, 136
- Poliomyelitis, paralysis in a dog simulating, 272
- Poliomyelitis, passive human carriage of virus of, 131
- Poliomyelitis, virus of, insect transmission of, 98
- Post-mortem examination of horses' hearts from cases of auricular fibrillation, 579
- Potentialdifferenzen, bioelektrischen, physikalische Natur der, 386
- R**EACTION of the lungs to the intrabronchial insufflation of killed virulent pneumococci and of plain sterile bouillon, 301
- Respiration, effective artificial, in emergencies, simple devices for, 306
- ROBINSON, G. CANBY. The influence of the vagus nerves on the faradized auricles in the dog's heart, 554
- ROUS, PEYTON. False transitions between normal and cancerous epithelium, 235
- ROUS, PEYTON, and MURPHY, JAMES B. Variations in a chicken sarcoma caused by a filterable agent, 138
- ROUS, PEYTON, MURPHY, JAMES B., and TYTLER, W. H. A filterable agent the cause of a second chicken tumor, an osteochondrosarcoma, 30
- S**ALVARSAN and neosalvarsan, arsenic absorption following intramuscular injections of, 541









